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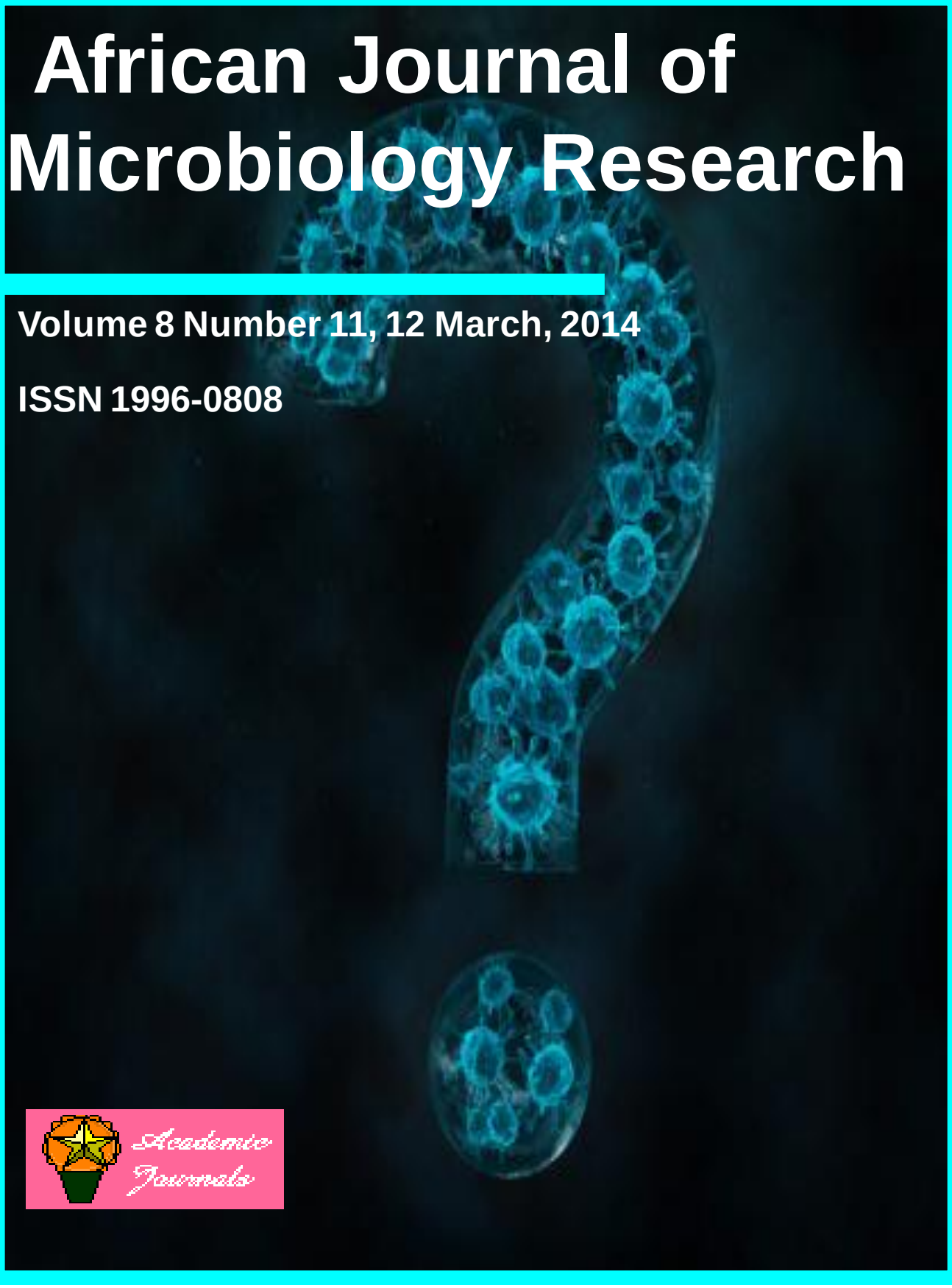

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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Full Length Research Paper

Plant growth promoting rhizobacteria (PGPR) induces resistance against *Fusarium* wilt and improves lycopene content and texture in tomato

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Plant growth promoting *Bacillus subtilis* (BS2) was found effective against tomato wilt caused by *Fusarium oxysporum* f sp. *lycopersici* under field conditions. Pretreatment of tomato plants with *B. subtilis* BS2 significantly induced the activities of defense related enzymes *viz*., peroxidase, polyphenol oxidase, chitinase and phenylalanine ammonialyase and phenolics when challenged with the pathogen. Apart from disease control, BS2 improved the fruit quality with high lycopene (76.30 mg/kg against control, 40.34 mg/kg) and texture (90.5 F_{max} against control, 56.35 F_{max}) during harvest and even 15 days after harvest, similar trend was maintained unequivocally indicating that plant growth promoting rhizobacteria (PGPR) can improve the nutritional quality as well as shelf life of the fruits.

Key words: Plant growth promotion, plant growth promoting rhizobacteria (PGPR), defense enzymes, lycopene, fruit texture.

INTRODUCTION

Tomato is an important worldwide cultivated solanaceous vegetable playing a major role in human nutrition. Wilt caused by *Fusarium oxysporum* f.sp. *lycopersici* is a serious concern in tomato cultivation (Loganathan et al., 2009). Management of the disease through an eco-friendly approach will nullify the ill effects such as environmental pollution, residual toxicity and fungicidal resistance in pathogens due to use of chemicals. Many bacterial communities living in the rhizosphere and found associated with plant growth promotion activity are called plant growth promoting rhizobacteria (PGPR) (Kloepper et al., 1980). The PGPR are known to act as biocontrol agents through competition for ecological niche/substrate and production of siderophores, antibiotics, hydrogen cyanide and fungal cell wall lysing enzymes (Glick and Bashan 1997; Wang et al., 2000; Saravanakumar et al., 2007). PGPR are also involved in induced systemic resistance (ISR) against many diseases in a wide range of crops (Kloepper and Beauchamp, 1992; Liu et al., 1995; Chen et al., 2000; Sangeetha et al., 2010). Although the application of PGPR and their effects on disease or pest control and yield have been well

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established for a wide range of crops, information on fruit quality in terms of lycopene content and texture is very limited. Lycopene, a carotenoid present in tomato is an important compound in human nutrition as it plays a key role in reducing cardiovascular as well as prostate cancer diseases (Giovannucci, 1999; Giovannucci et al., 2002). Apart from the medicinal value, the market quality of the tomato fruit is also affected by lycopene because fruit color is determined by carotenoids, and primarily lycopene (Fraser et al., 1994; Shi and Le Maguer, 2000). Shelf life of tomato fruits is dependent on fruit firmness. Main factor affecting firmness of the fruit during maturity is microbial spoilage (Kramer et al., 1992). In our earlier study we identified two potential PGPR: *Bacillus amyloliquefaciens* isolate BA1 (earlier reported as chilli 1) and *Bacillus subtilis* isolate BS2 (earlier reported as chilli 2), based on preliminary studies related to plant growth promotion and plant disease control (Loganathan et al., 2010). In that study, BA1 and BS2 manifested high level of *in vitro* efficacy in producing plant growth promoting compounds (indole acetic acid and siderophores) and solublizing phosphates apart from inhibiting mycelial growth of wilt fungus, *F. oxysporum* fsp. *lycopersici*. In the present study, field efficacy of PGPR bio-formulations (BA1 and BS2) on *Fusarium* wilt and quality of fruits (lycopene content and texture) during harvest and post-harvest were determined.

MATERIALS AND METHODS

Testing the talc bio-formulation efficacy under field conditions

Talc formulation of PGPR isolates BA1 and BS2 were prepared using a standard protocol (Vidhyasekaran and Muthamilan, 1995) with some minor modifications. The PGPR isolates were cultivated in nutrient broth (HiMedia Laboratories Pvt. Ltd., Mumbai, India) for 48 h at temperature of 28±2°C under shaking at 20 g. Four hundred milliliters of bacterial culture containing 8 x 10^8 cfu/ml was mixed with 1 kg talc (Talc India, Rajasthan, India) sterilized at 105°C for 12 h along with 10 g carboxy methyl cellulose (adhesive agent) and 15 g calcium carbonate (to neutralize the pH to 7.0). The bio-formulation was shade dried for 12 h at ambient temperature (28±2°C) and packed in polythene packs. Bacterial population in the talc bio-formulation at the time of application was 6 x 10^8 cfu/g. Two field trials were conducted in years 2009 (trial I at Upland Block at Indian Institute of Vegetable Research, Varanasi, India) and 2010 (trial II at Lowland Block at Indian Institute of Vegetable Research, Varanasi, India). Both blocks have recorded 40-60% *Fusarium* wilt incidence in tomato (cv DVRT-1) in the previous year. The seeds of tomato (cv. DVRT-1 obtained from Seed Production Unit, Indian Institute of Vegetable Research, Varanasi, India) were treated with the talc formulation (10 g formulation/kg of seeds) and shade dried for 12 h. The treated seeds were sown in nursery beds applied with talc formulation (mixed thoroughly 50 g talc formulation with 5 kg well decomposed Farm Yard Manure (FYM) and applied to the 3m^2 bed). In chemical treatment, seeds treated with 1 g of carbendazim 50% WP (Dhanuka Agritech Ltd., Gurgaon, India) per kg of seeds were sown in nursery beds applied with well decomposed FYM (5 kg/3m^2). In control, nursery beds were prepared as described in chemical treatment but seeds were sown without any treatment. After 25 days, under bio-formulation treatment, main field was applied with 2.5 kg bioformulation in 50 kg of well decomposed FYM ha^{-1}. The seedlings under bio-formulation treatment were uprooted and the root portion was dipped in talc based bio-formulation solution (1.0%) for 30 min and transplanted in the main field. Similarly, for the chemical and untreated controls, the seedlings were dipped for 30 min in 0.1% carbendazim solution and water respectively and transplanted separately in the main field and FYM alone (50 kg/ha) was applied. The experiment was conducted in randomized block design with four replications of each treatment. Observations on disease control and measurement of plant height were recorded 45 days after transplanting.

Testing quality of fruits

In all the treatments the mature fruits were harvested from the field separately and analyzed for fruit texture and lycopene content. In tomato, third harvest normally gives maximum yield since it matches with exact maturity. Hence third harvest fruits were used in all fruit quality analyses. For each of these analyses, five fruits were randomly collected from each replication (in each treatment there were four replications hence total number of fruits were 20/treatment).

Analysis of fruit texture

Texture of the fruit was analyzed by a puncture test using a Texture Analyzer TA-XT (Stable Micro Systems), loading (2 mm s^{-1}) at a distance of 15 mm on two opposite points along the equatorial plane (Mena-Violante and Olalde-Portugal, 2007). Finally an average maximum force (F_{max}) per fruit was calculated.

Estimation of lycopene

Lycopene content was estimated by a spectrophotometric method as described by Anthon and Barrett (2001). The samples were extracted with a solution containing ethanol and hexane (4:3 v/v) and the phases were separated. Amount of light absorption were recorded from hexane phase at 503 nm and the results were interpreted using the value of 172/nM as the extinction coefficient for lycopene in hexane and expressed in mg/kg of fruits

Sample collection for analyses of induced defense proteins and phenolics

Pot mixture (consists of red soil : sand : well decomposed FYM at 1:1:1 ratio) was autoclaved for 1 h for two consecutive days at 24 h interval and filled in pots (20 cm diameter and 30 cm height). Virulent isolate of *F. oxysporum* f.sp. *lycopersici* (FOL) was mass multiplied in sterilized sand-maize (19:1 ratio) medium for 15 days and mixed in the pot soil (10 g sand maize fungal culture/kg of soil). Tomato seeds were treated with talc formulation as described in field experiments and sown on sterilized soil. After 24 days, the seedlings were uprooted and the root portions of the seedlings were dipped in 1.0% talc formulation solution for 30 min. The treated seedlings were transplanted into the pot containing sterilized pot mixture treated with PGPR talc formulation (10 g/kg of soil) with or without pathogen according to the treatment details mentioned in Table 5. For analysis of defence related enzymes and chemical, root portion of 45 days old seedlings (21 days after transplanting) from different treatments was used. Five seedlings per replication were uprooted and thoroughly washed with running tap water. Root portions were removed from the seedlings and cut into pieces and powdered using liquid nitrogen and the frozen powder was used for each analysis. The protein content of sample was estimated by Lowry et al. (1951) using bovine serum albumin

as standard.

Assay of phenylalanine ammonia-lyase (PAL)

One gram of plant root sample was homogenized in 3 ml of ice cold 0.1 M sodium borate buffer (pH 7.0) containing 1.4 mM of 2-mercaptoethanol and 50 mg of insoluble polyvinylpyrrolidone (PVP) by using pestle and mortar. The extract was filtered through cheese cloth to remove debris, and centrifuged at 20,000 *g* for 10 min at 4°C and the supernatant was used as the enzyme source. PAL activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm (Dickerson et al., 1984) and was expressed in nmol trans-cinnamic acid min^{-1} mg^{-1} of protein

Assay of chitinase

Plant root sample (1 g) was extracted with 5 ml of 0.1 M sodium citrate buffer (pH 5.0) and the content was centrifuged at 20,000 *g* at 4°C for 10 min and the supernatant was used as enzyme source. The colorimetric assay of chitinase was carried out as described by Boller and Mauch (1988) and the enzyme activity was expressed as nmoles GlcNAc equivalents min^{-1} mg^{-1} protein.

Assay of peroxidase (PO)

One gram of plant root sample was homogenized with 2 ml of 0.1 M phosphate buffer (pH 7.0) at 4°C and then centrifuged at 15,000 *g* and 4°C for 15 min. The supernatant (0.5 ml) was mixed with 0.5 ml of 0.0 5M pyrogallol and 0.5 ml of 1% H_2O_2 and incubated at room temperature (28 ± 2°C). The absorbance was recorded at 420 nm at 30 s intervals up to 3 min and the activity was expressed as changes in the absorbance min^{-1} mg $protein^{-1}$ (Hammerschmidt et al., 1982).

Assay of phenol

Root samples (1 g) were homogenized in 80% methanol as described by Zieslin and Ben-Zaken (1993). The reaction mixture consisting of 1 ml methanolic extract, 5 ml distilled water and 250 µl Folin-Ciocalteau reagent (1 N) was prepared and kept at 25°C. The development of blue colour was measured at 725 nm and compared with standard catechol and the amount of phenolics was expressed as µg catechol mg $protein^{-1}$.

Native PAGE analysis of polyphenol oxidase (PPO)

The effect of PGPR treatment on induction of PPO isoforms was visualized in native PAGE electrophoresis (Laemmli 1970). Root tissue (1 g) was homogenized in 2 ml of 0.1 M sodium phosphate buffer (pH 6.5) at 4°C and centrifuged at 20,000 g for 10 min at 4°C and the supernatant was used as enzyme extract. Sample (60 µg) containing protein was loaded in polyacrylamide gel (8%). After electrophoresis, the gel was equilibrated in 0.1% *p*-phenylene diamine in 0.1 M potassium phosphate buffer (pH 7.0) for 30 min followed by 10 mM catechol in the same buffer. Finally, the PPO isoforms were visualized by adding catechol which resulted in appearance of dark brown discrete bands (Jayaraman et al., 1987).

Statistical analysis

Data were statistically analyzed (Gomez and Gomez,1984) and the treatment means were compared by Duncan's multiple range test (DMRT). The package IRRISTAT version 92 developed by the International Rice Research Institute Biometrics unit, Philippines was used in all the analyses.

RESULTS

Efficacy of PGPR isolates against *Fusarium* wilt disease of tomato under field conditions

In both trials (trials I and II), *B. amyloliquefaciens* BA1 and *B. subtilis* BS2, demonstrated substantial wilt reduction as compared to the untreated control, and served as good or better wilt control than the chemical treatment. Comparing the two isolates, BS2 showed significantly better field efficacy against FOL than BA1 in the trials (Table 1). In trial I, BS2 treatment has less incidence of wilt as compared to BA1, chemical and control treatments (16.0, 25.3, 26.0 and 45.2%, respectively; $P<0.05$). Similarly in trial II, BS2 showed significantly less wilt incidence (24.6%) and the effect was statistically at par with the chemical control (23.8%) and better than BA1 (28.0%). Pooled mean comparison of both trials revealed that BS2 registered 58.4% wilt reduction over control which was greater than the chemical control (48.97%). Observations on yield parameters revealed that BS2 recorded the highest yield (43.90 t/ha) followed by BA1 (38.50 t/ha) and chemical (32.5 t/ha) while the least quantity was recorded in control (26.85 t/ha) (Table 2). The yield parameter had a positive relationship with plant growth as it was highest in BS2 (57.7 cm) followed by BA1 (51.95 cm), chemical (48.15 cm) and control (44.80 cm).

PGPR treatment on quality of fruits

Fruits collected at different harvesting periods were analyzed for lycopene content and texture (Tables 3 and 4). Lycopene content was increasing invariably towards maturity of the plants irrespective of the treatments. However, under PGPR treatments the content was significantly higher than in the chemical and untreated control. Among the treatments, BS2 recorded the highest content in all the harvest (71.28 mg/kg) when compared with BA1 (46.21 mg/kg), chemical (39.12 mg/kg) and control (35.21 mg/kg). Apart from this, fruits were analyzed for texture and lycopene content at the time of harvest and after 15 days of harvest. Results indicate that in all cases, there was a decline in texture profile after 15 days of storage while lycopene content increased (Table 4). At harvest, the highest texture profile (F_{max}) was recorded in plants inoculated with BS2 (90.5) followed by BA1 (79.16), carbendazim (61.96) and control (56.35). After 15 days, there was a declining trend in F_{max} value in all the treatments but in BS2 the content was high (11.10) as compared to control (2.14) and other

Table 1. Effect of PGPR treatment on fusarial wilt disease of tomato under field conditions.

Treatment	**Wilt incidence (%)		Mean wilt incidence (%)	Wilt reduction over control (%)
	Trial I	Trial II		
BA-1	25.30[b]	28.00[b]	26.65	45.39
BS-2	16.00[a]	24.60[a]	20.30	58.40
Carbendazim	26.00[b]	23.80[a]	24.90	48.97
Control	45.20[c]	52.40[c]	48.80	0.00

Means in a column followed by the same superscript letters are not significantly different according to Duncan's multiple range test at P=0.05. **Data were transformed using arc-sine and analyzed

Table 2. Effect of PGPR on plant growth and yield of tomato under field conditions.

Treatment	Plant height^ (cm)		Mean	*Yield (t/ha.)		Mean
	Trial I	Trial II		Trial I	Trial II	
BA-1	56.3[b]	47.6[b]	51.95	45.00[b]	32.00[b]	38.50
BS-2	62.1[a]	53.3[a]	57.70	49.20[a]	38.60[a]	43.90
Carbendazim	50.0[c]	46.3[b]	48.15	36.00[c]	29.00[c]	32.50
Control	47.6[c]	42.0[c]	44.80	32.10[d]	21.60[d]	26.85

^Plant height measured during the 3rd harvest; *Cumulative yield of three harvests; Means in a column followed by same superscript letters are not significantly different according to Duncan's multiple range test at P=0.05.

Table 3. Effect of PGPR treatment on lycopene content at different stages of fruit harvest.

Treatment	Lycopene content (mg/kg)			Mean
	First harvest	Second harvest	Third harvest	
BA-1	45.00[b]	46.00[b]	47.63[a]	46.21
BS-2	64.10[a]	73.44[a]	76.30[a]	71.28
Carbendazim	37.00[c]	37.36[c]	43.00[b]	39.12
Control	30.30[d]	35.00[c]	40.34[b]	35.21

Means in a column followed by same superscript letters are not significantly different according to Duncan's multiple range test at P=0.05.

Table 4. Effect of preharvest application of PGPR on post harvest fruits quality.

Treatment	Texture profile analysis (TPA) force (F_{max})		Lycopene content (mg/kg)	
	At harvest*	15 days after harvest	At harvest*	15 days after harvest
BA-1	79.16[b]	7.86[b]	47.63[b]	48.34[b]
BS-2	90.50[a]	11.10[a]	76.30[a]	82.70[a]
Carbendazim	61.96[c]	4.23[b]	43.00[c]	48.65[b]
Control	56.35[d]	2.14[c]	40.34[d]	44.70[c]

*Fruits collected from 3rd harvest were used; Means in a column followed by same superscript letters are not significantly different according to Duncan's multiple range test at P=0.05.

treatments. Post harvest lycopene analysis indicated that there was an increase in the content after 15 days of storage in all the treatments (Table 4) but the increase was greater in BS2 treatment (82.70 mg/kg) followed by carbendazim (48.65 mg/kg), BA1 (48.34 mg/kg) and control (44.70 mg/kg).

Table 5. Induction of defense related enzymes and phenol in tomato plants challenged with FOL.

Treatment	Phenylalanine mmonia lyase (nmol of transcinnamic acid min^{-1} mg^{-1} of protein)	Peroxidase (Changes in the absorbance min^{-1} mg^{-1} of protein)	Poly phenol oxidase (Changes in the absorbance min^{-1} mg^{-1} of protein)	Chitinase (nmol of GlcNAc mg^{-1} min^{-1})	Phenol (µg catechol mg of protein)
BA1	23.33^{d}	1.13^{c}	1.63^{c}	5.61^{b}	43.00^{b}
BS2	25.60^{c}	1.00^{c}	1.40^{d}	4.73^{bc}	43.00^{b}
BA1+FOL	29.10^{b}	1.90^{a}	1.84^{b}	9.71^{a}	58.64^{a}
BS2+FOL	30.53^{a}	1.96^{a}	2.60^{a}	11.30^{a}	59.30^{a}
FOL	22.00^{d}	1.63^{b}	1.50^{d}	6.33^{b}	45.63^{b}
Healthy plant	20.00^{e}	0.80^{d}	1.30^{f}	3.00^{c}	42.30^{b}

Values are the means of three replicates; Means in a column followed by same superscript letters are not significantly different according to Duncan's multiple range test at P=0.05.

Induction of defense related enzymes

Induction of phenyl alanine ammonia lyase (PAL), peroxidase (PO) polyphenol oxidase (PPO), chitinase and phenol was observed in plants treated with PGPR upon challenged with FOL (Table 5). Induction of PAL was more in PGPR treated plants challenged with FOL and the effect was greater in BS2 (30.53 nmol min^{-1} mg^{-1}) followed by BA1 (29.10 nmol min^{-1} mg^{-1}), pathogen inoculated control (22.00 nmol min^{-1} mg^{-1}) and untreated control (20.00 nmol min^{-1} mg^{-1}). Similarly, all PGPR treated plants challenged with pathogen showed high level induction of PO, chitinase and phenol as compared to the control. However, in the case of PPO, the induction was significantly high in BS2 treated plants challenged with pathogen followed by BA1 and other treatments.

Native PAGE analysis of tomato plants treated with BS2 with or without pathogen inoculation showed four isoforms (PPO1, PPO2, PPO3 and PPO4) in BS2 treated plants challenged with FOL (Figure 1). Among the four isoforms, PPO3 and PPO4 were induced commonly in healthy control, pathogen inoculated control and BS2 with or without pathogen treated plants. On the other hand, PPO2 was absent in healthy and present in the other treatments and PPO1 was exclusively present in BS2 treated plants challenged with pathogen. Presence of strong signal of PPO3 and PPO4 isoforms was observed in the BS2 treated plants challenged with pathogens.

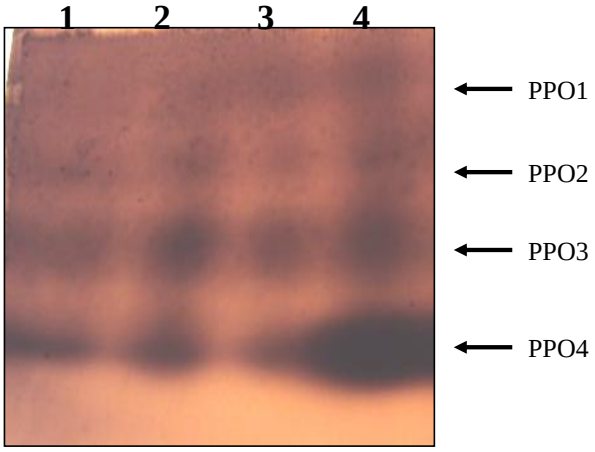

Figure 1. Induction of PPO isozyme in tomato plant treated with BS2 treatment with or without FOL, where lanes 1, Healthy; 2, FOL inoculated control; 3, BS2 treatment alone; 4, BS2 treatment challenged with FOL.

DISCUSSION

Majority of PGPR isolated for the management of plant diseases are *Pseudomonas* spp., (Ramamoorthy et al., 2002) or *Bacillus* spp., (Schisler et al. 2004). In our preliminary study, out of 142 rhizospheric PGPR tested, two *Bacillus* spp.: *B. amyloliquefaciens* and *B. subtilis* were identified as potential isolates for use against fungal pathogens such as *F. oxysporum* f.sp. *lycopersici* and *Sclerotium rolfsii* apart from plant growth promotion through production of indole acetic acid and phosphate solubilization (Loganathan et al., 2010). In the present study, among the two isolates (BS2 and BA1), BS2 proved to be more effective against wilt pathogen, *F. oxysporum* f.sp. *lycopersici* under field conditions. Similarly, PGPR were reported to control fungal diseases caused by soil borne and leaf spot pathogens in wide range of crops (Wei et al., 1996; Viswanathan and Samiyappan, 2001; Ramamoorthy et al., 2002; Vivekananthan et al., 2004).

In the present study, the lycopene content in DVRT1 was found to increase from first harvest onwards and reached maximum at the third harvest. However, the influence of PGPR treatment, especially BS2 on lycopene content was greater since in all three harvests, the content was significantly higher than other treatments indicating that BS2 has a pivotal role in enhancing the content. It has been reported that environment conditions and plant nutrient status played a major role on status of lycopene content (Abushita et al., 2000; Binoy et al.,

2004). By keeping environmental conditions constant, there was a clear difference between PGPR treated and untreated plant fruits indicating the direct or indirect influence or both on lycopene content. Apart from lycopene, important quality of fruit is shelf life or post harvest keeping quality. Fruits from the BS2 treated plot exhibited considerably high level texture even at well ripening stage (15 days after harvest). Fruits with high texture showed extended keeping quality. The fruit texture is associated with low production ethylene production (Alexander and Grierson, 2002) and it has been demonstrated in plants treated with PGPR (Glick et al., 1998). In the present study, alternation of ethylene production is indirectly reflected in promoting growth of the tomato plants (Table 2). Though there is a relationship between PGPR treatment and improvement of fruit texture, further research in this line is required. This has been found to be of significant importance since less textured/firm fruits are more prone to spoilage by microbes (Mena-Violante and Olalde-Portugal, 2007).

Application of PGPR for plant disease management is gaining importance, due to induction of defense related enzymes (Viswanathan and Samiyappan, 2001) and chemicals (Chen et al., 2000) to suppress the pathogen thereby enabling a process called induced systemic resistance (ISR). In the present study, PAL, PO, PPO, chitinase and phenol were triggered in PGPR treated plant inoculated with FOL indicating activation of ISR mechanism by PGPR. The defense related enzymes play a major role in biosynthesis of lignin, oxidation of phenol and synthesis of antimicrobial phytoalexins (Daayf et al., 1997). The defense reaction was observed in several crops against a wide range of pathogens. Tomato plants treated with *Pseudomonas fluorescens* Pf1 recorded high level induction of defence enzymes in response to *Pythium aphanidermatum* attack (Ramamoorthy et al., 2002). Similarly, mango trees sprayed with *P. fluorescens* FP7 recorded a greater amount of the enzymes against *Colletotrichum gloeosporioides* infection (Vivekanadan et al., 2004). Mixture of *Pseudomonas* (NFP6), *P. fluorescens* (Pf3a), and *B. subtilis* (BS1) was found to induce defence reaction in banana against crown rot caused by *Lasiodiplodia theobromae* and *Colletotrichum musae* under *in vivo* conditions (Sangeetha et al., 2010). Phenolics are known to have fungitoxic effect and a role in strengthening the host cell wall. PGPR treated plants showed high level accumulation of phenolic compounds when challenged with pathogens (M'Piga et al., 1997). Similarly, pre-treatment with PGPR had shown induction of phenolics against *F. oxysporum* f. sp. *pisi* and *Pythium ultimum* in pea and cucumber, respectively (Benhamou et al., 1996, 2000). In the present study, among the different defense related enzymes, PPO was found to be induced significantly in BS2 treated plants challenged with FOL. Similarly in native PAGE, induction of new or enhanced accumulation of PPO in tomato pretreated with BS2 against FOL indicated that PPO also played a crucial role in protecting the crop from the soil borne pathogen. Ramamoorthy et al. (2002) also noted unique induction of PPO in native PAGE analysis in PGPR treated tomato and chilli against *P. aphanidermatum*.

In conclusion, induction of defense related enzymes and phenolics by *Bacillus* PGPR, was found to have protective role in combating the wilt disease caused by *F. oxysporum* f.sp. *lycopersici* in tomato which in turn improved the fruit yield and quality.

Conflict of interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Assessment of five phenotypic tests for detection of methicillin-resistant staphylococci in Cotonou, Benin

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The study aimed at assessing performance and cost of phenotypic tests for detecting methicillin resistance in *Staphylococcus* spp. isolates in Cotonou, Benin. Isolates consecutively collected from various specimens from four medical laboratories in Cotonou from December 2012 to April 2013 were included in the study. The isolates were subjected to five phenotypic tests: disk diffusion tests with cefoxitin (Cefox) and moxalactam (Moxa) on Mueller Hinton agar incubated at 37°C, oxacillin on Mueller Hinton agar incubated at 30°C (Oxa30), oxacillin on salt Mueller Hinton agar incubated at 37°C (Oxa37) and agglutination test for PBP2a detection (TPBP 2a). Results were compared with polymerase chain reaction (PCR) of *mec*A gene which was used as the gold standard. In addition, cost per reagent of each phenotypic test was assessed. Considering the general agreement with PCR, Cefox and Moxa were the best tests in *S. aureus* while in non-*aureus Staphylococcus* isolates, TPBP 2a was the best test but its cost was 20 times higher than that of disk diffusion tests.

Key words: *Staphylococcus* spp., methicillin resistance, diffusion disk tests, PBP 2a, *mec*A.

INTRODUCTION

Staphylococcus spp. has been recognized as one of the most frequent bacteria isolated in routine laboratory practice. Even though, *Staphylococcus aureus* is the most pathogen among the staphylococci, non-*aureus* species commonly called “coagulase negative staphylococci” have become increasingly important in human pathology due to various reasons among which is the rising prevalence of immunocompromised patients, particularly in sub-Saharan Africa (Adeyemi et al., 2010; Reddy et al., 2010).

Staphylococcus spp. can show resistance to several antibiotics. Of particular importance in clinical practice is the case of methicillin resistance in which almost all the β-lactams are inactive against this pathogen (Gould et al., 2012). Due to their affordability, low toxicity and high efficacy in treating common diseases, β-lactams are

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among the most prescribed groups of antibiotics in medical practice. Therefore, rapid and accurate detection of methicillin resistance in infections caused by *Staphylococcus* species is of paramount importance.

Methicillin resistance is mediated by the production of an altered penicillin-binding protein (PBP), 2a coded by the *mec*Agene complex (Dumitrescu et al., 2010). Detection of *mec*A gene by polymerase chain reaction (PCR) is the most accurate method for detecting methicillin resistant *Staphylococcus* spp. isolates (Akpaka et al., 2008; Majouri et al., 2007; Mohanasoundaram et al., 2008). However, the use of this molecular assay is largely restricted to reference centres in developed countries, and this test is not currently available in most routine diagnostic laboratories, particularly in resource constraints settings. Indeed, PCR for *mec*A gene is expensive and in its basic form includes many steps such as DNA extraction, amplification and electrophoresis. When it is used in the form of Real Time PCR, it is shorter but also expensive to use in resource-limited settings.

In low-income countries like Benin, only phenotypic methods are used for detecting methicillin resistant isolates (Seydi et al., 2004; Affolabi et al., 2012). These include disk diffusion tests with either cefoxitin (Cefox) or moxalactam (Moxa) on Mueller Hinton (MH) agar incubated at 37°C, oxacillin on MH agar incubated at 30°C (Oxa30) and oxacillin on salt MH agar incubated at 37°C (Oxa37). It is also possible to detect PBP 2a in isolates using a commercial agglutination test.

Several studies have evaluated phenotypic tests, however these studies were either performed in developed countries or did not use comparison, an appropriate reference method such as the detection of *mec*A gene. In addition, most of these studies were restricted only to *S. aureus* while non-*aureus Staphylococcus* species were not included. Furthermore, cost of tests which is an important parameter in developing countries, was not also assessed (Mohanasoundaram et al., 2008; Olowe et al., 2013).

The present study was carried out in Cotonou, Benin to evaluate five phenotypic tests for detecting methicillin resistant *Staphylococcus* spp. using PCR-based *mec*A gene as a gold standard.

MATERIALS AND METHODS

Bacterial isolates

Isolates were collected from Medical Microbiology Laboratories of the University Teaching Hospital, Hubert Koutoukou Maga (the reference hospital for the country), Saint Luc Hospital, Menontin Hospital and that of the Ministry of Health, Cotonou. The first three laboratories receive specimens from outpatients as well as inpatients while the latter receives specimens mainly from non-hospitalized patients. All isolates were Gram-positive cocci occurring in pairs or clusters, have grown on mannitol salt agar and were catalase-positive. Differentiation between *S. aureus* from non-*aureus Staphylococcus* species was done using PCR-amplification of the *nuc* gene (Brakstad et al., 1992).

Phenotypic tests

Disk diffusion tests

After preparation of 0.5 McFarland standard suspension of each isolate, a 1:10 dilution of the suspension was inoculated on MH agar plate (Biorad, France) supplemented with or without 4% NaCl as recommended by the Antibiogram Committee of the French Society of Microbiology (AC-FSM, 2012). Antibiotic disks (Biorad, France) were applied on the plate and incubation was done at 37°C aerobically for 24 h. The inhibition zone diameter for each isolate was measured and compared with interpretative standards (AC-FSM, 2012).

Detection of PBP 2a (TPBP 2a)

PBP 2a was detected using a commercial agglutination kit (Oxoid, United Kingdom) according to the manufacturer's instructions. Briefly, protein extraction was carried out by heating a heavy bacterial suspension at 100°C for 3 min. After addition of the extraction reagent, the mixture was centrifuged at 4,500 rpm for 5 min and the supernatant (the extract) collected. Then, the test and the control reagents were mixed with the extract for 3 min and agglutination was then observed. As recommended by the manufacturer, for non-*aureus* species, only colonies around the oxacillin disk on MH agar plate were used since a PBP 2a induction is needed for these species prior detection.

mecA gene amplification by PCR

DNA extraction

DNA extraction from each isolate was carried out as previously described (Mayoral et al., 2005). Briefly, colonies were emulsified in 500 µl of sterile distilled DNA-free water. The mixture was boiled at 100°C for 15 min, cooled on ice and then centrifuged at 13,000 rpm for 5 min. The supernatant containing the DNA was stored at 4°C before use.

DNA amplification

It was carried out as previously described by Majouri et al. (2007) with minor modifications on amplification program. Primers used were *mecA1*: *5'-GTAGAAATGACTGAACGTCCGATAA-3'* and *mecA2*: *5'-CCAATTCCACATTGTTTCGGTCTAA-3'* (Eurogentec, Belgium). The 50 µl mix reaction contained 200 µM for each dNTP (Sigma, USA); 1X enzyme buffer (Sigma, USA); 0.4 µM of each primer, 1.25 U of Jump Start Taq polymerase (Sigma, USA) and 5 µl of DNA extract. PCR amplification program was as follows: initial denaturation at 94°C for 5 min, 37 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min and 30 s, extension at 72°C for 1 min and final extension at 72°C for 10 min. The size of the final amplification product was 310 bp.

Quality control

Methicillin resistant *S. aureus* strains ATCC 43300 and a well-characterized methicillin susceptible *S. aureus* were used as positive and negative controls, respectively for phenotypic as well as molecular tests. In addition, for molecular tests, standard microbiological procedures were strictly followed in order to

Table 1. Characteristics of disk diffusion tests studied.

Disk	Incubation temperature	Medium	Critical diameter (mm)		
			R	I	S
Cefoxitin (30 µg)	37°C	MHA	< 25	25-27	≥ 27
Moxalactam (30 µg)	37°C	MHA	< 23	23-24	≥ 24
Oxacillin (5 µg)	37°C	Salt MHA	< 20		≥ 20
Oxacillin (5 µg)	30°C	MHA	< 20		≥ 20

MHA: Mueller Hinton agar; R: resistant; I: intermediate; S: susceptible; salt MHA: MHA supplemented with 4% NaCl.

Table 2. Comparison between phenotypic tests and PCR of *mec*A gene.

Disk		PCR *mec*A					
		S. aureus			Non-*aureus Staphylococcus*		
		Positive	Negative	Total	Positive	Negative	Total
Cefoxitin	R	27	01	28	43	03	46
	S	01	86	87	06	49	55
Moxalactam	R	27	01	28	43	05	48
	S	01	86	87	06	47	53
Oxacillin 30°C	R	22	00	22	19	03	22
	S	06	87	93	30	49	79
Oxacillin37°C	R	23	00	23	38	02	40
	S	05	87	92	11	50	61
PBP2a	R	27	03	30	48	02	50
	S	01	84	85	01	50	51

R: Resistant; S: susceptible.

minimize cross contamination. DNA extraction and PCR-amplification were done in molecular laboratories that were separated from the routine clinical microbiology laboratory. The PCR laboratory has designated sections for pre-amplification, DNA extraction and amplification/post-amplification with a unidirectional movement of staff.

Cost assessment of reagents per test

While one technician was performing a test, a second technician recorded the quantity of reagents used. Prices of reagents collected from a local supplier were presented in US dollars (US$) (change rate on 15th December, 2013). Only reagents were taken into account for cost calculation. Some reagents were prepared in batches and the whole cost for the batch was first recorded. In the final assessment, the cost for the test was calculated by taking into account the portion of the batch used for the test.

Data analysis

Data were entered and analysed using Excel software. The sensitivity, specificity and the agreement of each phenotypic test were calculated using PCR of the *mec*A gene as gold standard with the formulas:

Sensitivity = [True Positive/(True Positive + False Negative)] × 100
Specificity = [True Negative/(True Negative + False Positive)] × 100
Agreement = [(True Positive + True Negative)/Total strains tested] x 100

RESULTS

A total of 216 *Staphylococcus* spp. isolates (*S. aureus*, N=115; non-*aureus Staphylococcus*, N=101) were recovered from various clinical specimens. Of these, 127 (58.80%) were urine samples, 53 (24.54%) were wound swabs, 30 (13.89%) were genital fluid, while 6 (2.78%) were blood cultures.

Table 1 summarizes characteristics of each test performed. Results of the comparison between phenoltypic tests and PCR of *mec*A gene are shown in Table 2, while Table 3 shows performance of each phenotypic test. Of all the phenotypic tests performed, the best agreement with the gold standard (98.26%) was

Table 3. Performances of phenotypic tests.

Test	*S. aureus*			Non-*aureus Staphylococcus*		
	Sensitivity (%)	Specificity (%)	Agreement (%)	Sensitivity (%)	Specificity (%)	Agreement (%)
Céfoxitin	96.43	98.85	98.26	87.76	94.23	91.09
Moxalactam	96.43	98.85	98.26	87.76	90.38	89.11
Oxacillin 30°C	78.57	100	94.78	38.78	94.23	67.33
Oxacillin 37°C	82.14	100	95.65	77.55	96.15	87.13
PBP 2a	96.43	96.55	96.52	97.96	96.15	97.03

Table 4. Performances of tests combinations

Combination	*S. aureus*			Non *aureus Staphylococcus*		
	Sensitivity	Specificity	Agreement	Sensitivity	Specificity	Agreement
A	82.14	100	95.65	77.55	94.23	86.14
B	96.43	98.85	98.26	87.76	92.31	90.1
C	96.85	98.85	98.26	87.76	90.38	89.11
D	96.43	96.55	96.52	97.96	92.31	95.05
E	96.43	98.85	98.26	87.76	94.23	91.09
F	96.43	98.85	98.26	87.76	90.38	89.11
G	96.43	96.55	96.52	97.96	94.23	96.04
H	96.43	98.85	98.26	89.80	88.46	89.11
I	96.43	96.55	96.52	97.96	92.31	95.05
J	96.43	96.55	96.52	97.96	88.46	93.07

A : Oxacillin 30°C + oxacillin 37°C; B: oxacillin 30°C + cefoxitin; C: oxacillin 30°C + moxalactam; D: oxacillin 30°C + PBP2a; E: oxacillin 37°C + cefoxitin; F: oxacillin 37°C + moxalactam; G: oxacillin 37°C + PBP2a; H: cefoxitin + moxalactam; I: cefoxitin + PBP2a; J : moxalactam + PBP2. The final result of a combination was considered as resistant if at least one the test showed a resistant result, otherwise it was considered as susceptible.

obtained with Cefox and Moxa for *S. aureus* isolates while for non-*aureus* strains, TPBP 2a had the best agreement, 97.03% (Table 3). Although, Oxa30 and Oxa37 had the lowest agreement rates of 78.57 and 82.14% respectively when compared with gold standard, they had the highest specificities (100%) (Table 3).

The results of performance of combining phenotypic tests are presented in Table 4. The final result of a combination was considered as resistant if at least one of the tests showed a resistant result; otherwise, it was considered as susceptible. For *S. aureus* isolates, the best agreement of 98.26% was obtained with the following tests combinations: Oxa30 + Cefox; Oxa30+Moxa; Oxa37+Cefox; Oxa37+Moxa and Cefox+Moxa while for non-*aureus* isolates, Oxa37 + TPBP 2a showed the best agreement (96.04%) with PCR-based *mec*A gene (Table 4).

In addition, two non-*aureus Staphylococcus* isolates showed methicillin resistance in all diffusion disk tests done while PCR for *mec*A gene and TPBP 2a were negative. Concerning the cost of reagents per phenotypic test, the cost of all diffusion disk tests was close and varied from US$ 0.57 to 0.64, while TPBP 2a was 20 times more expensive than diffusion disk tests (Table 5).

DISCUSSION

Medical bacteriology laboratories in most French speaking countries in sub-Saharan Africa usually follow recommendations from the French Society of Microbiology without performing their own studies to determine the suitability of use of such recommendations in their settings (Seydi et al., 2004; Affolabi et al., 2012; AC-FSM, 2012). In order to test these recommendations in Cotonou (Benin), we compared five phenotypic tests for detecting methicillin resistant *Staphylococcus* spp. isolates (*S. aureus* as well as non-*aureus Staphylococcus*) using PCR-based *mec*A gene as gold standard.

We observed that oxacillin disk diffusion tests had the lowest performances. This finding is in agreement with other studies (Boutiba-Ben Boubaker et al., 2004; Majouri et al., 2007; Datta et al., 2011; Olowe et al., 2013). Despite these similar findings, these tests are still in the 2012 recommendations of the French Society of

Table 5. Reagents cost per test.

	Test				
	Cefoxitin	**Moxalactam**	**Oxacillin, 37°C**	**Oxacillin, 30°C**	**PBP 2a**
Reagents cost/ test (US $)	0.57	0.57	0.64	0.57	11.20

Microbiology and are being used in several laboratories in sub-Saharan African countries like Benin (Seydi et al., 2004; Affolabi et al., 2012). One of the advantages of oxacillin disk diffusion tests is their high specificity but their sensitivity is low for recommendation in routine work. Other disadvantages of these tests are the need to use a salt MH agar (for Oxa37) or an incubation temperature of 30°C (for OXA 30). This incubation temperature is not readily accessible in several medical laboratories in low-income countries.

From this study, Cefox and Moxa were found to be the best phenotypic tests for detecting methicillin resistance in *S. aureus* as each of them (Cefox and Moxa) had the highest performance agreement of 98.26% when compared with the gold standard (Table 3). This is in agreement with submissions of other workers within the region and elsewhere (Boutiba-Ben Boubaker et al., 2004; Majouri et al., 2007; Datta et al., 2011; Olowe et al., 2013) where Cefox and Moxa have been recommended for use in detection of methicillin resistance in *S. aureus*-based infections. These tests even performed better than TPBP 2a in *S. aureus* (Table 3), reinforcing the need of using one of these tests in our routine diagnostic laboratories.

For non-*aureus Staphylococcus* isolates, Cefox and Moxa still performed well but the best test was TPBP 2a (Table 3). Only few studies have evaluated methicillin resistance detection tests in non-*aureus Staphylococcus* isolates (Majouri et al., 2007; Souza Antunes et al., 2007). In fact, these species are often considered as contaminants when isolated from clinical samples. However, it is now well known that *Staphylococcus saprophyticus* is a common cause of urinary tract infection ("honey-moon cystitis") in immunocompetent sexually active women (Raz et al., 2005). Furthermore, in immunocompromised patients, non-*aureus Staphylococcus* species can cause severe infections such as blood stream infections and endocarditis (Adeyemi et al., 2010; Reddy et al., 2010). As these species often yield resistance to several groups of antibiotics, thus an accurate identification of methicillin resistance is of utmost importance for correct management of such infections. In this study, we observed that the best test was TPBP 2a. The test is rapid; time to get result is less than 30 min while antibiotics disk diffusion tests require 24 h to give results. However, TPBP2a is too expensive (about 20 times the cost of diffusion disks tests) (Table 5) to be routinely used in laboratories in low resource countries. In spite of this limitation, TPBP 2a could be proposed for severe infections due to non-*aureus Staphylococcus* species in reference laboratories even in resource-limited settings. If TPBP2a is not available, Cefox and Moxa can be used as their performances are quite good even in non-*aureus Staphylococcus* species, as seen in this study (Table 5).

A surprising observation in this study was a result from two non-*aureus Staphylococcus* isolates that showed methicillin resistance in all diffusion disk tests performed while PCR for *mec*A gene and TPBP 2a were repeatedly negative. Even though, primers used in the present study were from a highly conserved region of *mec*A gene, false negative PCR results cannot be excluded (Geha et al., 1994). Similar observations were made for *S. aureus* in Switzerland, United Kingdom and Denmark (García Álvarez et al. 2011; Monecke et al., 2013). This may be due to a new variant of *mec*A, recently named *mec*C, which codes for a new protein (PBP 2c). Further investigations are needed to confirm this finding in non-*aureus Staphylococcus* isolates.

In conclusion, Cefox and Moxa are were found to be the best phenotypic tests to detect methicillin resistance in *S. aureus* isolates but in non-*aureus Staphylococcus* isolates, TPBP 2a is the best although 20 times more expensive than diffusion disks tests.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Bacteria from infected surgical wounds and their antimicrobial resistance in Hawassa University Referral Teaching Hospital, Southern Ethiopia

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A study was carried out from November 2010 to June 2011 in Hawassa University Referral Teaching Hospital to identify bacterial species involved in post-operative wound infections and to determine their antimicrobial resistance pattern. The study involves 100 surgical patients with post-surgical wound infections. Swab samples of wound discharge were collected for bacteriological examination and inoculated on appropriate culture media. Isolates were identified and characterized by standard methods and antibiotic resistance was determined using the Kirby-Bauer disk diffusion method. A total of 177 bacterial isolates were identified in the study. The most dominant isolates were *Staphylococcus aureus*, *Klebsiella* spp., *Escherichia coli* and coagulase negative staphylococci (CoNS) accounting for 45 (25.4%), 32 (18.1%), 30 (16.9%) and 26 (14.7%) of the isolates respectively. Other bacteria isolated include *Pseudomonas aeruginosa* (9.0%), *Proteus* spp. (6.8%), Streptococci (5.1%), *Citrobacter* spp. (2.3%) and *Enterobacter* spp. (1.7%). Of the 177 isolates, 173 (97.7%) were resistant to at least 1 antimicrobial, while 164 (92.7%) were resistant to ≥2 antimicrobials. Resistance of isolated organisms was 76.3% to amoxicillin, 71.2% to penicillin, 56.9% to vancomycin, 39.5% to ceftriaxone and norfloxacin and 31.1% to gentamicin. The susceptibility of *S. aureus* was 64.4% to gentamicin but it was 100% resistant to amoxicillin. All isolates of *P. aeruginosa* were resistant to penicillin and amoxicillin. The rate of resistance of *S. aureus* to 2 or more antimicrobials was 97.8% and that of *P. aeruginosa* was 100%. This study confirms that the bacteria commonly implicated in post-operative wound infections: *S. aureus*, *Klebsiella*, *E. coli*, CoNS, and *P. aeruginosa*, continued to dominate and have developed high level of drug resistance to some important antibiotics. Periodic surveillance of the species of bacteria involved in post-operative wound infection and determination of their antimicrobial resistance is recommended for empirical treatment.

Key words: Antimicrobial resistance, post-operative, surgical, wound infection, Ethiopia, Hawassa.

INTRODUCTION

Despite advances in infection control, surgical site infections (SSIs), formerly called surgical wound infections, remain a substantial cause of morbidity and mortality among hospitalized patients (Mangram et al., 1999). They were the 3rd most frequently reported nosocomial infection, accounting for 14 to 16% of all nosocomial infections among hospitalized patients in the US (Emori and Gaynes, 1993). Surgical wound infection increases hospital stay (Poulsen et al., 1994; Mitt et al., 2005) and thereby the cost of medical treatment (Boyce et al., 1990; Kirkland et al., 1999). It doubles the patient's risk of death after surgery (Kirkland et al., 1999)

and causes patient discomfort. It also places a significant burden on the health system (Poulsen et al., 1994), especially in Africa where resources are limited.

Staphylococcus aureus is the predominant bacterial pathogen isolated from post-operative wound infections. Other bacteria frequently associated with post-operative wound infection include coagulase negative staphylococci (CoNS), *Enterococcus* spp., *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterobacter* spp., *Klebsiella pneumoniae*, *Proteus mirabilis* and *Streptococci* spp. (Mangram et al., 1999; Hidron et al., 2008).

Post-operative wound infection risk depends on a number of factors. Those most frequently cited in the literature include length of surgical procedure, surgical procedure category, obesity, use of pre-operative prophylactic antibiotics, colonization with microorganisms, age, sex, anemia, diabetes mellitus, malnutrition, smoking and length of pre-operative hospitalization (Barber et al., 1995; Anvikar et al., 1999; Ahmed et al., 2007). The virulence and invasive capability of the organisms influence the risk of infection, but the physiological state of the tissue in the wound and immunological integrity of the host also have importance in determining whether infection occurs (Fry, 2003).

Wound infection is most commonly characterized by the classic signs of redness (rubor), pain (dolor), swelling (tumor), elevated incisional tissue temperature (color) and systemic fever. Ultimately, the wound is filled with necrotic tissue, neutrophils, bacteria and proteinaceous fluid that together constitute pus (Fry, 2003).

A high level of post-operative wound infection (17.9%) was reported from one of the largest hospitals in Ethiopia, with significant proportion of isolates showing drug resistance (Tekie, 2008). However, there is no adequate data on the problem in the country in general and in the study area in particular, in spite of the necessity of periodic review of isolates and their drug resistance patterns for effective prevention and treatment of surgical wound infections.

This study, therefore, aimed to find out bacterial species involved in post-operative wound infections and to determine their antimicrobial resistance pattern in Hawassa University Referral Teaching Hospital.

MATERIALS AND METHODS

The study area

The study was conducted at Hawassa University Referral Teaching Hospital (HURTH) from November 2010 to June 2011. Hawassa town is located at 7°5' Latitude N and 38° 29' longitude E, 275 km south of Addis Ababa, the capital of Ethiopia. It is the capital of Southern Nations Nationalities and Peoples Region. HURTH serves as a main referral center for the Southern part of Ethiopia serving roughly 10 million people in the region and surrounding areas. The hospital went operational in 2003 and is affiliated to the Hawassa University. There are a total of 400 beds in the hospital. However currently only 250 are in use; of which 58 are surgical.

Patients' profile

The study involved 100 patients, 37 males and 63 females, who underwent surgical operations for various reasons and developed post-operative surgical wound infection in the study period. Eighty one of the patients underwent emergency surgery while 19 were operated on elective bases. The study included surgical patients from general surgery, pediatrics and obstetrics and gynecology wards. The age of the patients ranged from 1 to 77 years.

Sample collection and transportation

Samples for bacteriology, from apparently infected surgical wounds, were collected aseptically with sterile cotton tipped swabs. The wounds were examined for suggestive signs of infection: redness, pain, swelling, elevated tissue temperature, discharge and systemic fever (Fry, 2003) before the patients were discharged from the hospital.

Examination for suggestive signs and symptoms of infection and specimen collection was carried out by experienced nurses working in the respective wards. When infection was clinically suspected, the area around the surgical wound was cleaned with 70% ethyl alchohol. The exudates were collected from the depth of the wound using sterile cotton tipped swabs. All the specimens collected were immediately transported to the microbiology laboratory of the hospital for bacteriological examination. Samples were inoculated onto appropriate culture media as soon as they arrived at the laboratory.

Isolation and identification

The samples were inoculated on blood agar, mannitol salt agar (selective medium for *S. aureus*) and MacConkey agar (Oxoid, Basingstoke, England). The plates were incubated at 37°C for 24 - 48 h aerobically. All positive cultures were identified by their characteristic appearance on their respective media, Gram staining reactions and confirmed by the pattern of biochemical reactions using the standard methods (Cheesbrough, 2006). Members of the family *Enterobacteriaceae* and other Gram-negative rods were identified by indole and H_2S production, gas formation, citrate utilization, motility and oxidase and urease test. For Gram-positive bacteria coagulase and catalase tests were used (Cheesbrough, 2006).

Antimicrobial susceptibility testing

Antimicrobial susceptibility tests were done on Mueller-Hinton agar (Oxoid, Basingstoke, England) using Kirby-Bauer disk

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Table 1. Frequency of isolation and percentage of the total number of isolates of bacteria from post-operative wound infections (n=100).

Bacterial isolate	No. of isolates	Percentage (%)
S. aureus	45	25.4
Klebsiella spp.	32	18.1
E. coli	30	16.9
CoNS[a]	26	14.7
P. aeruginosa	16	9.0
Proteus spp.	12	6.8
Streptococcus spp.	9	5.1
Citrobacter spp.	4	2.3
Enterobacter spp.	3	1.7
Total	177	100

[a]Coagulase negative staphylococci.

diffusion method (NCCLS, 2003) for six antimicrobials currently used in the hospital setting and available on the market. Morphologically identical 4-6 colonies from overnight culture were touched with sterile loop and inoculated in 5 ml nutrient broth and incubated for 4 h at 37°C. Turbidity of the broth culture was matched to 0.5 McFarland standards. The surface of Mueller-Hinton agar plate was evenly inoculated with the culture using a sterile cotton swab. The antibiotic discs were applied to the surface of the inoculated agar using sterile forceps. After 18-24 h of incubation, the diameter of growth inhibition around the discs were measured using a millimeter scale to the nearest millimeter and interpreted as sensitive, intermediate or resistant according to National Committee for Clinical Laboratory Standards (NCCLS) (2003). The antimicrobial agents tested were: amoxicillin (Aml), norfloxacin (Nor), vancomycin (Van), gentamicin (Gen), penicillin (Pen) and ceftriaxone (Cro) (Oxoid, Basingstoke, England).

Data analysis

The collected data were entered in MS Excel and analyzed using Stata version 9 for windows (Stata Corp. College Station, TX). Sex and age of the patient, type of surgery (elective or emergency), and ward (surgical, obstetrics and gynecology and pediatrics) were considered as potential factors which could affect the prevalence of a specific bacteria in post-operative wound infection. Association of these factors with the frequency of bacterial type (species/genus) isolated was analyzed using univariable logistic regression analysis.

Ethical consideration

The study obtained ethical clearance from the Institutional Review Board of Hawassa Collage of Medicine and Health Sciences. Patients, or parents in case of children, were told about the objectives of the study and those who gave their informed consent were included in the study.

RESULTS

Bacterial isolates

Of the 100 samples collected from infected post- operative wounds, 92 (92%) were positive for aerobic culture. *S. aureus* was the most prevalent isolate with 45 (45%) samples positive followed by *Klebsiella* spp. (32%), *E. coli* (30%), coagulase negative staphylococci (CoNS) (26%), *P. aeruginosa* (16%), *Proteus* spp. (12%), *Streptococcus* spp. (9%), *Citrobacter* spp. (4%) and *Entrobacter* spp. (3%). Out of the 92 culture positive samples, the majority (80) were polymicrobial, that is, yielded 2 to 3 isolates. A single species/genus of bacteria was isolated from 13 samples while 73 samples resulted in recovery of 2 isolates. Three isolates were recovered only from 6 samples. All polymicrobial infections in our study involved Gram positive and Gram negative bacteria with *S. aureus* and *Klebsiella* species being the most common association in 16 cases. Other frequent associations were: *S. aureus* and *E. coli* (10 cases), *S. aureus* and *P. aeruginosa* (9 cases), CoNS and *Klebsiella* spp. (8 cases) and CoNS and *E. coli* (7 cases).

A total of 177 bacterial isolates were recovered from the 100 samples examined. Out of these 177 isolates, 97 (54.8%) were Gram negative bacteria while the rest 80 (45.2%) were Gram positive. The proportion of each bacterial isolate to the total number of isolates is presented in Table 1. The most dominant isolates were *S. aureus, Klebseilla* spp., *E. coli* and CoNS accounting for 25.4, 18.1, 16.9 and 14.7% of the total isolates, respectively.

Effects of sex, age, type of surgery and ward on the types and frequency of bacterial isolates

The results of univariable analyses of frequency of isolation of bacteria from infected post-operative wounds with possible risk factors are summarized in Table 2. Frequency of isolation of *Klebsiella* and *Pseudomonas* were significantly associated (P <0.05) with gender of the surgical patient. *Klebsiella* was most frequently isolated from female patients (41.3 vs. 16.2%) while *Pseudomonas* predominated in male patients (29.3% Vs 7.9%).*Staphylococcus aureus* tended to be more prevalent in females (52.4% Vs 32.4%) but the difference just failed to be significant (P =0.053). There was, however, no significant association (P >0.05) between sex of the patient and the frequency of other isolates recovered in the study.

Age of the patient and type of surgery (elective or emergency) appeared to have no significant association (P >0.05) with the frequency of isolation of any type of bacteria identified in the study.

Samples from patients with post-operative wound infection in gynecology and obstetrics ward resulted in a significantly (P <0.05) high *S. aureus* isolation than samples from pediatrics and surgery wards. Similarly, samples from gynecology and obstetrics ward yielded a significantly (P <0.05) more *Klebsiella* as compared to

Table 2. Association of frequency of bacterial isolates, from infected post-operative wounds, with sex, age, type of surgery and ward.

Factor		Sex		Age			Type of surgery			Ward	
Group		**Male**	**Female**	**0-14**	**15-60**	**≥61**	**Elective**	**Emergency**	**G&O[a]**	**Pediatrics**	**Surgical**
	No.	12	18	6	18	6	3	27	10	6	14
E. coli	%	32.4	28.6	26.1	29.5	37.5	15.8	33.3	27.0	26.1	35.0
	P value	0.684			0.740		0.170			0.670	
	No.	4	8	3	8	1	3	9	5	3	4
Proteus	%	10.8	12.7	13.0	13.1	6.3	15.8	11.1	13.5	13.0	10.0
	P value	0.779			0.827		0.694			0.858	
	No.	12	33	10	28	7	8	37	25	10	10
S. aureus	%	32.4	52.4	43.5	45.9	43.8	42.1	45.7	67.6	43.5	25.0
	P value	0.053			0.975		0.778			0.001	
	No.	6	26	8	20	4	7	25	17	8	7
Klebsiella	%	16.2	41.3	34.8	32.8	25.0	36.8	30.9	45.9	34.8	17.5
	P value	0.010			0.868		0.615			0.027	
	No.	11	5	3	11	2	2	14	4	3	9
Pseudomonas	%	29.3	7.9	13.0	18.0	12.5	10.5	17.3	10.8	13.0	22.5
	P value	0.004			0.862		0.730			0.415	
	No.	0	4	1	2	1	1	3	1	1	2
Citrobacter	%	0.0	6.3	4.3	3.3	6.3	5.3	3.7	2.7	4.3	5.0
	P value	0.294			0.789		0.576			0.872	
	No.	2	7	4	5	0	1	8	2	4	3
Streptococcus	%	5.4	11.1	17.4	8.2	0.0	5.3	9.9	5.4	17.4	7.5
	P value	0.478			0.212		0.527			0.321	
	No.	2	1	1	1	1	0	3	0	1	2
Enterobacter	%	5.4	1.6	4.3	1.6	6.3	0.0	3.7	0.0	4.3	5.0
	P value	0.553			0.336		0.528			0.446	
	No.	12	14	4	15	7	6	20	5	4	17
CoNS[b]	%	32.4	22.2	17.4	24.6	43.8	31.6	24.7	13.5	17.4	42.5
	P value	0.261			0.195		0.538			0.011	

[a]Gynecology and obstetrics; [b]Coagulase negative staphylococci.

samples from surgical ward. However, post-operative infected wounds from surgical ward (42.5%) had a higher prevalence of CoNS as compared to wounds in obstetrics and gynecology (13.5%) and pediatric (17.4%) wards (P <0.05).

Antimicrobial resistance

Antimicrobial susceptibility of all isolates recovered in this study is summarized in Table 3. All the 177 bacterial isolates obtained from infected surgical wounds were tested for 6 antimicrobials. Of the 177 isolates belonging to 9 species/genera, 173 (97.7%) were resistant to one or more antimicrobials, while 164 (92.7%) were resistant to 2 - 6 antimicrobials.

The overall resistance of isolated organisms, irrespective of species/genus, was 76.3% to amoxicillin, 71.2% to penicillin, 56.9% to vancomycin, 39.5% to ceftriaxone and norfloxacin each and 31.1% to gentamicin.

All of the 45 *S. aureus* isolates were resistant to 1 or more antimicrobials and 44 (97.8%) were resistant to ≥2 antimicrobials. Resistance of *S. aureus* was detected for all the 6 antibiotics tested in this study. All the 45 isolates (100%) were resistant to amoxicillin, while 30 (66.7%) were resistant to vancomycin. However, 29 (64.4%) were susceptible to gentamicin.

Resistance was also high in *Klebsiella* isolates: 30 (93.8%) being resistant to 1 or more antibiotics and 23 (71.9%) to 2 or more antimicrobials. The highest resistance of *Klebsiella* (78.1%) was recorded against amoxicillin. All the 30 *E. coli* isolated in this study were resistant to 2 or more antimicrobials and all of them were resistant to penicillin. On the other hand, all of them were susceptible to norfloxacin and gentamicin. Of the 26 CoNS 24 (92.3%) were resistant to ≥2 antimicrobials. Resistance to penicillin among CoNS isolates was 92.3%. All the 16 *Pseudomonas* isolates had resistance

Table 3. Number (percentage) of antimicrobial resistant isolates of bacteria from surgical wound infections.

Isolate	Antibiotics					
	Cro	Nor	Gen	Van	Pen	Aml
S. aureus (n=45)	16 (35.6)	23 (51.1)	9 (20.0)	30 (66.7)	16 (35.6)	45 (100)
Klebsiella (n=32)	9 (28.1)	9 (28.1)	12 (37.5)	12 (37.5)	18 (56.2)	25 (78.1)
E. coli (n=30)	10 (33.3)	0 (0)	0 (0)	20 (66.7)	30 (100)	20 (66.7)
CoNS (n=26)	13 (50)	13 (50)	13 (50)	4 (15.4)	24 (92.3)	4 (15.4)
P. aeruginosa (n=16)	8 (50)	10 (62.5)	8 (50)	14 (87.5)	16 (100)	16 (100)
Proteus spp. (n=12)	8 (66.7)	8 (66.7)	6 (50)	10 (83.3)	11 (91.7)	11 (91.7)
Streptococcus spp. (n=9)	2 (22.2)	4 (44.4)	5 (56.6)	4 (44.4)	4 (44.4)	7 (77.8)
Citrobacter spp. (n=4)	2 (50)	1 (25)	0 (0)	3 (75)	4 (100)	4 (100)
Enterobacter spp. (n=3)	2 (66.7)	2 (66.7)	2 (66.7)	2 (66.7)	4 (100)	4 (100)
Total isolates (N =177)	70 (39.5)	70 (39.5)	55 (31.1)	99 (56.9)	126 (71.2)	135 (76.3)

Cro (ceftriaxone), Nor (norfloxacin), Gen (gentamicin), Van (vancomycin), Pen (penicillin), Aml (amoxicillin).

to ≥2 antimicrobials and all of them were resistant to penicillin and amoxicillin. Only 4 (25.0%) isolates of *P. aeruginosa* were found susceptible to at least 1 of the antibiotics tested and it was only to gentamicin. Resistance to 2 or more antimicrobials was 100% in *Proteus* spp., and resistance to penicillin, amoxicillin and vancomycin was 91.7, 91.7 and 83.3% respectively. Eight (88.9%) of the 9 streptococci were resistant to ≥2 antimicrobials and 7 (77.8%) isolates were resistant to amoxicillin and 5 (56.6%) to gentamicin. All *Citrobacter* and *Enterobacter* spp. isolated in this study were resistant to penicillin and amoxicillin. However, no *Citrobacter* was resistant to gentamicin.

DISCUSSION

Bacterial isolates

Out of the 100 samples collected from post-operative wound infections, 92 (92%) were culture positive yielding a total of 177 bacterial isolates. Similarly, Rao and associates (2013) recovered bacterial isolates from 96 of 100 samples collected from clinically suspected post-operative wound infections in India. A Nigerian study reported isolation rate of 96.2% from infected wounds (Sule et al., 2002). The absence of bacterial growth in samples collected from apparently infected surgical wounds could be due to the effect of antimicrobials which are used routinely in surgery, it can also be due to antiseptics used for cleaning the wounds or it may even be due to the body's defense mechanism overcoming the infection. It is also possible that some organisms were unable to grow under the aerobic condition the samples were cultured. Anaerobic bacteria (Mangram et al., 1999; Rao et al., 2013) and *Candida albicans* (Mangram et al., 1999; Hidron et al., 2008) were implicated in some wound infections in the literature.

The bacteria isolated in our study were commonly associated with surgical wound infections in Ethiopia and elsewhere (Ahmed et al., 2007; Tekie, 2008; Shanthi et al., 2012; Rao et al., 2013). Similar to our observation (25.4%) *S. aureus* was reported to be the most dominant isolate from surgical wound infections representing 25-45.1% of the total isolates (Ahmed et al., 2007; Anguzu and Olila, 2007; Khorvash et al., 2008; Tekie, 2008; Shanthi et al., 2012). The high prevalence of *S. aureus* infection may be partly explained by the presence of these bacteria in the nose, skin and intestinal tract of human beings (Vandepitte et al., 2003). With the disruption of natural skin barrier, *S. aureus* which are common bacteria on surfaces, easily find their way into surgical sites.

Klebsiella spp. (18.1%) and *E. coli* (16.9%) were the 2nd and 3rd most frequent isolates in our study which is consistent with the report of Khorvash et al. (2008) from Iran. A Nigerian study indicated *Klebsiella* spp. (25.3%) as the most dominant isolate from wound infections (Sule et al., 2002). Anvikar and associates (1999) reported *K. pneumoniae* as the most common isolate from clean surgical wounds. A study conducted in India reported *E. coli* as the most common isolate (20.8%) followed by *S. aureus* and *Pseudomonas* species (Rao et al., 2013).

In our study *Pseudomonas* was high in males than females. This finding agrees with the report from India by Ruhil et al. (2009) and Ranjan et al. (2010) where the prevalence of *P. aeruginosa* was higher in male patients, as compared to the females. A similar result was reported from Addis Ababa, Ethiopia, (Tekie, 2008) where the rate of *P. aeruginosa* infection was relatively higher in males than in females.

In this study, the highest proportions of *S. aureus* and

Klebsiella spp. were isolated from gynecology and obstetrics wards. Our result was in agreement with a report from Nigeria by Sule et al. (2002) where *Klebsiella* species were found to be the most common in wounds from obstetrics and gynecology units.

In our study, highest proportion of CoNS was isolated from general surgery ward as compared to other wards. A similar result has been reported from a teaching hospital in Addis Ababa, Ethiopia, (Tekie, 2008) where CoNS were recovered with high rate from surgical emergency ward.

Antimicrobial resistance

All the *S. aureus* isolates were resistant to amoxicillin while the majority of *S. aureus* (64.4%) were sensitive to gentamicin. This finding was similar to results of Anguzu and Olila (2007) where the majority of the *S. aureus* isolates (87.5%) were sensitive to gentamicin. The sensitivity of *S. aureus* to gentamicin recorded in a Nigerian study (60.0%) was also in agreement with our result (Nwachukwu et al., 2009). In the same Nigerian study, 10 out of 20 (50%) isolates of *S. aureus* were sensitive to norfloxacin, supporting our finding of 51.1% resistance. The resistance of *P. aeruginosa* to gentamicin was 50.0% in exact agreement with the resistance level recorded in a study performed by Nwachukwu and associates (2009) in Nigeria.

In conclusion this study indicated that *S. aureus*, *Klebsiella, E. coli*, CoNS and *P. aeruginosa* were the most important bacteria responsible for post-operative wound infection in Hawassa University Referral Teaching Hospital. High rates of drug resistance to some commonly used antibiotics were observed in this study and this warrants attention to the problem. Resistance was especially high in *S. aureus, P. aeruginosa* and *Proteus* spp.

Periodic surveillance of bacterial species involved in post-operative wound infections and their antimicrobial resistance is recommended to minimize the incidence of post-operative wound infections, shorten duration of recovery and reduce cost of medical treatment through empirical treatment which takes into account the epidemiological information on the species of bacteria involved and their antimicrobial resistance.

Conflict of interests

The author(s) have not declared any conflict of interests.

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Qualitative and quantitative study on bacterial flora of farm raised common carp, *Cyprinus carpio* in India

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Analysis of pond water and sediment as well as skin and intestine of common carp, cultured under polycultre system, was done quantitatively and qualitatively. During the study of 60 days (winter and summer seasons), total viable counts of bacteria were in the range of 4.43±0.50x10^3 to 5.5 ±0.09x10^3 cfu g^{-1} and 7.43±0.03x10^3 to 9.66±0.09x10^3 cfu g^{-1}, respectively in water of A, B and C ponds. In the sediment, bacterial biomass during winter was in the range of 3.23 ±0.06x10^4 to 4.46± 0.15x10^4 cfu g^{-1} and during summer it was found to be in the range of 8.3 0.26x10^4 cfu g^{-1} to 9.43± 0.24 x10^4 cfu g^{-1}. During winter and summer phase, bacterial biomass in skin and intestine was 3.16 0.09 x10^3 cfu g^{-1} to 3.56 0.12x10^3 cfu g^{-1} and 6.03 0.20 x10^5-10^6 cfu g^{-1} to 7.76 0.20 x10^5-10^6 cfu g^{-1} respectively for all 3 replicates. In total, 10 bacterial genera and 13 dominant species were identified. The bacteria of pond water and sediment reflected the bacterial composition in skin, gill and intestine of the fish. In the pond water, *Corynebacterium* spp., *Aeromonas hydrophila, Pseudomonas* spp., *Achromobacter* sp. and *Flavobacter* spp. were predominant whereas in pond sediment, *Aeromonas hydrophila, Pseudomonas* spp*., Corynebacterium* sp., *Flavobacter* spp. and *Bacillus* sp. were predominant and *Corynebacterium* spp., *Aeromonas hydrophila, Flavobacter* spp. and *Pseudomonas* spp. were predominant in skin, gills and intestine of common carp. During the experimental period, *Pseudomonas aeruginosa, Aeromonas hydrophila, Flavobacter devorans and Corynebacterium* sp. were predominantly present in all the samples in all the phases.

Key words: Polyculture, common carp, total viable counts, bacteria sediment, water.

INTRODUCTION

Bacteriology is one of the most important areas determining the pond dynamics and health and hygiene of fish farming system. The present day fish farming is based on nutritive feeds in addition to other management practices thus the bacteriology of cultured fishes in the tropics is receiving greater attention since some species of bacteria associated with fish cause diseases under stress condition. Bacteria within pond environment inhabit the water phase, the bottom sediment and of course live on plants, animals and detritus. Fish is in direct contact

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Table 1. Bacteria biomass (cfu × 10^3) ± S.D. in pond water.

Day	Winter phase			Summer phase		
	Pond A	Pond B	Pond C	Pond A	Pond B	Pond C
0	5.13±0.12	5.2±0.1	4.66±0.59	8.4±0.34	9.26±0.09	7.76±0.24
15	5.20±0.20	5.0±0.21	5.03 ±0.32	8.56 ±0.11	9.33±0.09	7.83 ±0.12
30	4.93± 0.35	4.96± 0.15	4.43± 0.50	8.26± 0.09	8.96± 0.09	7.76± 0.13
45	5.06± 0.24	5.26±0.12	5.26± 0.28	8.16± 0.36	8.86± 0.06	7.43 ±0.03
60	5.1 ±0.43	5.5± 0.09	5.36± 0.26	8.56± 0.20	9.66 ±0.09	7.96 ±0.26

with microflora in the environment and the opportunistic pathogens already present in the water invade the host under stress. There is growing awareness of the influence of bacterial composition of fish, especially in the intestine, on the health and growth of the host (Naim and Ahmed, 2012; Razavilar et al., 2013). Extreme examples of the influence of the gut flora include the negative effects on the pathogenic organisms. The influence of the gut flora on the host is clearly of great interest in aquaculture, particularly where poor productivity and/or stock losses are widespread (Skjermo and Vadstein, 1999; Lavens and Sorgeloos, 2000). The intestinal flora may be of significance in fish spoilage and faecal contamination spread (Al-Harbi, 2003). It is therefore, important to understand the microflora associated with fish culture environment. Recent interest on microbial study of aquaculture products also increases the importance of knowledge of microflora associated with fish (Reilly and Kaferstein, 1997). Bacterial load and bacterial type in shrimp and fish ponds have received attention of researchers recently (Otta et al., 1999; Ahmad and Naim, 2007) but little literature is available on the bacterial flora in cultivable fish (Cahill, 1990; Sugita, 2006). There is limited literature available on microbiological studies in fresh water fish and the culture environment. This study was done to evaluate the normal bacterial counts and identification heterotrophic bacteria found in common carp. The information will be of great value in determining whether there is need to control bacteriological parameters in farming system and also establish relationship between flora of gut of fish and culture water.

MATERIALS AND METHODS

The study was undertaken in the polyculture ponds of 0.3-0.4 ha with a depth of 1.5 m, in the Instructional Fish Farm at College of Fisheries, Pantnagar, India. The ponds were stocked with Catla (*Catla catla*), Rohu (*Labeo rohita*), Mrigal (*Cirrhinus mrigala*), Silver carp (*Hypophthalmichthys molitrix*), Grass carp (*Ctenopharyngodon idella*) and Common carp (*Cyprinus carpio*). The ponds of the farm were fertilized regularly and the fishes were daily fed with supplementary feed (rice polish, mustard oil cake and fish meal). Artesian tube well was used regularly to maintain water level. Two trials of 60 days each were conducted in two major seasons, that is, winter (December-January) and Summer (April - May). Soil and water sampling was done fortnightly. The samples were collected from three spots in the pond and mixed together. Water was analyzed for some important physico-chemical parameters using standard techniques (APHA, 2005). Skin and intestine samples were collected fortnightly, slaughtering three fishes at each sampling. The plate count method was used for quantitative estimation of aerobic heterotrophic bacteria from pond water, sediment and fish using plate count agar media (Breed et al., 1957). The bacterial isolates were identified to genera and species level by morphological studies, staining procedures and bio-chemical and physiological tests, that is, catalase test, acid and gas production from carbohydrates, starch hydrolysis, gelatin hydrolysis, decarboxylation of amino acids, indole production, Methyl Red and Voges-Proskauer (MRVP) test, urea hydrolysis and H_2S production (Breed et al., 1957). The data were analyzed by one way ANOVA (Panse and Sukhame, 1978). All the data were found significant at 5%.

RESULTS

The results of total bacterial counts carried out at 30±2°C for samples of experimental pond water, pond sediment and experimental fish skin and intestine are recorded in the Tables 1, 2, 3 and 4, respectively. It is clear from these values obtained from bacterial counts that samples are significantly different and observations showed seasonal variation too. The results of total plate count of pond water of pond A, during winter season, were found in the range of 4.93±0.35x10^3 to 5.2±0.2x10^3 cfu ml^{-1}; for pond B 4.90 ±0.15x10^3 to 5.5±0.09x 10^3 cfu ml^{-1} and 4.43±0.15x10^3 to 5.36 ±0.09x 10^3 cfu ml^{-1} for pond C (Table 1). During the summer phase counts of bacteria were in the range of 8.16±0.36x10^3 to 8.56±0.2x10^3 cfu ml^{-1} in pond A, 8.86±0.36x10^3 to 9.66±0.09x10^3 cfu ml^{-1} in pond B and 7.43±0.30x10^3 to 7.96±0.26x10^3 cfu ml^{-1} in pond C (Table 1). The heterotrophic populations in the sediment were in the range of 3.46±0.15x10^4 to 4.06±0.15x10^4 cfu g^{-1} in pond A, 3.80±0.27x10^4 to 4.46±0.15x10^4 cfu g^{-1} in pond B and 3.23±0.06x10^4 to 4.08±0.07x10^4 cfu g^{-1} in pond C (Table 2) during winter phase whereas during summer season, counts of bacteria were in the range of 8.3±0.26x10^4 cfu g^{-1} to 9.3±0.18 x10^4 cfu g^{-1} in pond A, 8.53±0.06x10^4 to 9.43±0.24x10^4 cfu g^{-1} in pond B and 8.23±0.09x10^4 to 8.53±0.22x10^4 cfu g^{-1} in pond C. Bacterial biomass on the body of the skin of the fish showed marked difference at the skin and in the intestine. Total counts were always higher in the intestine whereas skin bacterial counts were lower in the seasons, winter as well as summer. Table 3 shows that during winter season, the total viable counts of bacteria in

Table 2. Bacteria biomass (cfu × 10^4) ± S.D. in pond sediment.

Days	Winter phase			Summer phase		
	Pond A	Pond B	Pond C	Pond A	Pond B	Pond C
0	3.83 ±0.11	3.96 ±0.12	3.26 ±0.12	8.3± 0.26	8.53 ±0.06	8.13 ±0.09
15	3.53± 0.12	4.1± 0.12	3.45 ±0.12	8.6 ±0.06	9.2± 0.19	8.46 ±0.09
30	3.46 ±0.15	3.8 ±0.27	3.23 0.06	9.25± 0.15	9.25± 0.09	8.46 ±0.09
45	3.76 ±0.18	4.26 ±0.03	3.46 ±0.05	8.6 ±0.07	8.5 ±2.89	8.23 ±0.09
60	4.06 ±0.15	4.46± 0.15	4.08 ±0.07	9.3± 0.18	9.43± 0.24	8.53± 0.21

Table 3. Bacteria biomass in skin (cfu × 10^3 cm^{-2}) ± S.D. and intestine (cfu × 10^5- 10^6 g^{-1}) ±S.D. of common carp.

Days	Pond A		Pond B		Pond C	
winter phase	Skin	Intestine	Skin	Intestine	Skin	Intestine
0	3.23± 0.09	6.53± 0.22	3.32 ±0.06	7.76± 0.15	3.43±0.09	6.16±0.12
15	3.43± 0.18	6.76± 0.19	3.46 ±0.15	7.63 ±0.15	3.26±0.09	6.5 ±0.15
30	3.2 ±0.12	6.53 ±0.18	3.26± 0.12	7.6 ±0.23	3.16±0.06	6.03±0.20
45	3.3± 0.21	6.66 ±0.15	3.56± 0.12	7.66 ±0.33	3.43±0.12	6.53±0.13
60	3.46± 0.12	6.9± 0.12	3.73 ±0.03	7.76 ±0.20	3.56±0.12	6.6 ±0.12
Summer phase						
0	8.2 ±0.09	1.87 ±0.37	8.2± 0.09	2.19 ±0.07	7.93±0.09	1.85±0.04
15	8.33± 0.21	1.89± 0.48	8.33± 0.21	2.21± 0.01	8.33±0.09	1.87±0.02
30	8.0 ±0.15	1.85± 0.38	8.0 ±0.15	2.19 ±0.12	8.13±0.13	1.86±0.01
45	9.16 ±0.20	1.86± 0.37	9.16 ±0.20	2.16± 0.08	7.93± 0.1	1.82±0.03
60	9.53± 0.11	1.90 ±0.39	9.53 ±0.11	2.22± 0.01	8.4± 0.12	1.88 ±.03

Table 4. Percentage incidence of bacteria from pond water, sediment and fish (skin and intestine).

No. of isolates / Bacteria	Incidence (%)			
	90	118	60	78
	Water	Sediment	Skin	Intestine
Pseudomonas spp.	49	50	36	25
Aeromonas sp.	18	21	12	30
Flavobacter spp.	5	4	5	4
Achromobacter sp.	3	4	1	1
Corynebacteria spp.	3	5	2	3
Vibrio sp.	2	3	2	3
Proteus sp.	1	4	1	-
Micrococcus sp.	2	-	-	-
Bacillus sp.	-	3	-	-

the fish skin ranged from 3.2±0.12x10^3 to 3.46±0.12x10^3 cfu cm^{-2} for pond A, 3.26±0.12x10^3 to 3.73±0.03x10^3 cfu cm^{-2} for pond B and 3.16±0.06x10^3 to 3.56±0.12x10^3 cfu cm^{-2} for pond C. During summer season, bacterial load in the skin of fish (Pond A) ranged from 8.0±0.15x10^3 to 9.53±0.12x10^3 cfu cm^{-2}, 8.49±0.09x10^3 to 9.3±0.19x10^3 cfu cm^{-2} for pond B and 7.93±0.09x10^3 to 8.4±0.12x10^3 cfu cm^{-2} for pond C. The bacterial counts showed lower numbers in winter phase as compared to summer phase. During winter season, the total viable counts of bacteria in the fish intestine ranged from 6.53.2±0.18x10^5 to 6.9±0.12x10^5 cfu g^{-1} for pond A, 7.6±0.23x10^5 to 7.76±0.2x10^5 cfu g^{-1} for pond B and 6.03±0.20x10^5 to 6.60±0.12x10^5 cfu g^{-1} for pond C (Table 3). Total aerobic plate counts for intestine of fish in different ponds during summer phase were 1.85±0.38x10^6 to 1.9±0.39x10^6 cfu g^{-1} for pond A, 2.16±0.01x10^6 to

Table 5. Identity of the bacteria with source of isolation.

Source of sample	Identity of the bacteria
Pond water	*Corynebacterium* spp., *Pseudomonas aeruginosa*, *P. fluorescens*, *P. aureofasciens*, *Aeromonas hydrophila*, *Flavobacter devorans*, *Proteus* sp., *Micrococcus* sp.,
Pond sediment	*Corynebacterium* spp.,*Ps.aeruginosa*, *P.fluorescens*, *P.aureofasciens*, *Aeromonas hydrophila*, *Flavobacter devorans*, *Bacillus* sp., *Proteus* spp., *Achromobacter* sp.
Fish skin	*Corynebacterium* spp., *Aeromonas hydrophila*, *Flavobacter devorans*, *Achromobacter* sp., *Pseudomonas aeruginosa*, *Vibrio* sp., *Proteus* sp.
Fish intestine	*Corynebacterium* spp., *Aeromonas hydrophila*, *Flavobacter devorans*, *Pseudomonas aeruginosa*, *Vibrio* sp., *Achromobacter* sp.

$2.22\pm0.01x10^6$ cfu g^{-1} for pond B and $1.82\pm0.03x10^6$ to $1.88\pm0.03x10^6$ cfu g^{-1} for pond C. Bacterial profile of pond and fish (Table 4) reveal that *Pseudomonas* spp. and *Aerobacter* sp. were the dominant groups. In rearing water and pond sediment, *Pseudomonas* spp. was the dominant flora, comprising about 27-51% of the total population followed by *Aerobacter* sp. with 12-31% of the total population. The predominant organisms associated with the fish skin were the *Pseudomonas* group, which comprised 41 and 38% of the total bacteria observed. In the fish gut, *Aeromonas* sp. was dominant (31%) while *Pseudomonas* comprised 27% of the total isolates. The isolates from pond water were identified as *Corynebacterium* spp., *Aeromonas hydrophila, Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas aureofasciens, Vibrio sp., Flavobacter devorans, Proteus* sp. and *Micrococcus* sp. In pond sediment, *A. hydrophila, Pseudomonas* spp. *Corynebacterium* spp., *Bacillus* sp., *Flavobacter devorans, Achromobacter* sp. and *Proteus* sp. were present. In total, 11 species belonging to nine genera were identified from pond water, sediment and fish. In addition to the above bacterial species, *Vibrio* sp. was also observed in the fish (skin and intestine). The distribution of these bacteria in the five type of samples, that is, pond water, pond mud, skin, gill and intestine of fish are shown in Table 5.

DISCUSSION

Ayyappan and Pande (1989) reported the bacterial population in water phase in Indian ponds in the range of 0.12 to 4.65×10^3 no./ml. Das and Mukherjee (1999) found the bacterial counts in the range of 0.8-25.2 × 10^3 cfu/ml in polyculture ponds. The mean total viable counts of the pond water recorded, was 1.8x103-4.5x 10^3 cfu ml^{-1} during the study period in a semi intensive pond by Sharmila et al. (1996). In the present study, the bacterial load recorded for the two ponds were within the limits as reported by the previous workers. The heterotrophic bacterial population in the sediment was in the range of 0.79-26.67 × 10^4 nos/g in culture ponds of India, as reported by Ayyappan and Pandey (1989). Ayyappan and Pandey (1989) recorded total viale cell counts ranging from $1.0x10^5$ to $6.2x10^5$ cfu g^{-1} for the pond sediment. On the basis of the above findings, it can be stated that the level of sediment bacteria recorded in the present study was within the range. Bacterial density was higher during summer phase as compared to winter phase. This is obvious because of the ambient temperature leading to higher rate of metabolic activities and growth. Bacterial density was observed to be ten times higher at the pond sediment than in the water medium. This is expected because organic matter content is greater at the pond as the total solids of manure are suspended. Similar observations were recorded by other workers also (Glucai et al., 1992; Al-Harbi and Uddin 2004). Total bacterial counts were maximum in the intestine in all the three phases while skin showed minimum bacterial load. The observations are also supported by the findings of Ahmad and Naim (2007) where total bacterial load was in the range of $3.4\pm 1.8 \times 10^6$ to $5.8\pm0.4 \times 10^7$ cfu g^{-1} in the intestine and $7.1\pm 0.7x10^5$ to $8.7 \pm 1.1x10^6$ cfu g^{-1} in the gills. *Pseudomonas* spp. predominated in all the bacterial isolates examined from pond water and sediment. The finding is supported by previous reports (Fasanya et al., 1988; Das, 1991). *Aeromonas hydrophila* followed *Pseudomonas* in water and sediment (51 and 21% respectively in water and 46 and 19% respectively in the sediment). All the bacterial genera identified in skin, gill and gut were represented in water although in different percentages of incidence. Previous workers (Roberts, 1978; Sakata et al., 1980; Sugita et al., 1988; Das and Mukherjee, 1999; Ahmad and Naim Uddin, 2007) have reported that bacterial flora observed on surface, gill and intestine of pond reared fish/prawn are a reflection of bacterial flora of pond water. *P. fluorescens, Aeromonas hydrophila, Edwardsiella tarda, Vibrio* spp. and

Myxobacteria have been reported as normal microflora of water by Naim Uddin and Ahmed (2012) and Razavilar (2013) and can be found on body surface or in the intestinal tract of fishes (Gary, 2005). These bacteria under environmental stress produce epizootic outbreaks. According to Cristopher et al. (1978), Bacillus, *Corynebacteria, Flavobacter* and *Vibrio* are other dominant flora with *Pseudomonas*. *A. hydrophila* was the most dominant isolate in intestine and gills of common carp, with 31 and 20% incidence, respectively. This organism is one of the most opportunistic pathogens for fresh water fish as reported by Das and Mukherjee (1999). Aeromonads were the main etiological agents in disease outbreaks in India where several mortalities have been recorded (Kumar, 1989; Das, 1991; Nayak, 1993). During all the three phases, *P. aeruginosa, A. hydrophila, F. devorans* and *Corynebacteria* sp. were predominantly present in all the samples. Mesophilic aeromonads have been reported by several workers (Campbell and Buswell, 1983; Sugita et al., 1988) in wild and aquaculture freshwater fish and prawn. It is evident from this preliminary study that bacterial types isolated from *C. carpio* and its environment were all aerobic heterotrophic. Diverse genera of Gram negative bacteria were detected. The potential pathogenic nature of Gram negative isolates (*A. hydrophila, P. fluorescens* and *Vibrio* sp.) is beyond doubt as several reports support (Roberts, 1972; Sugita et al., 1988; Otta, 1999). It may be concluded that this study shows that there are several potential pathogens present in the environment and also as part of normal bacterial flora in Indian major carp common carp. Under stressful conditions, these organisms may become opportunistic and attack the body tissue and produce disease. The need is thus felt to monitor and regulate the bacterial parameters in the present aquaculture system where lot of management is done to enhance production.

Conflict of interests

The author(s) have not declared any conflict of interests.

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Salmonella spp. detection in chicken meat and cross-contamination in an industrial kitchen

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Chicken meat is a widely consumed food. However, broilers are implicated in contamination by *Salmonella* spp., since poultry is considered asymptomatic carrier of the pathogen. The objective of this study was to detect the presence of *Salmonella* spp. in chicken *in natura* and ready for consumption, as well as in the hands of employees, personal protection equipment and utensils in an industrial kitchen. In total, 18 *in natura* chicken samples, 18 cooked chicken samples and 30 surfaces were analyzed. Research was conducted in two stages: before and after the presentation of bacteriological analysis and the observational research for managers and employees of food preparation, for the discussion and changes in the procedures to handle and prepare chicken. *Salmonella* spp. was detected in 55.5% of *in natura* chicken in stage 1 and in 44.5% in stage 2. In cooked chicken, positive results were observed in 33.4 and 11.2% in stages 1 and 2. Concerning surfaces, the microorganism was detected in 40% (stage 1) and 53.3% (stage 2) of tested samples. The results show the occurrence of problems in the chicken processing chain, with evident cross-contamination, posing risks to the health of the end consumer.

Key words: Poultry, food safety, food microbiology, food contamination.

INTRODUCTION

The constant expansion of chicken meat trade at global level is due to the fact that this meat product has excellent nutritional value and relatively low production and processing costs. Chicken meat is rich in important nutrients such as proteins, lipids, vitamins and minerals. It enjoys considerable acceptance by consumers, and may be consumed by humans of all ages (Núcleo de Estudos e Pesquisas em Alimentação, 2011).

However, chicken meat is involved in the transmission of several pathogens that cause food-borne diseases that are important in public health. This high prevalence is due to the fact that these pathogens are distributed

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across the whole production chain, from birth of chickens to the end product. This contamination may be worsened by temperature and humidity conditions in farms. Among the most important pathogen present on chicken body surfaces is *Staphylococcus aureus*, while *Salmonella*, *Campylobacter* and *Escherichia* are the genera known to colonize the intestinal tract of broilers (Foley et al., 2008; Mendes, 2012).

The contamination of chicken meat by *Salmonella* spp. is the object of constant research and control in several countries due to the high prevalence, health risks to consumers and economic costs. *Salmonella* spp. is present in the environment, and around 2,300 different serotypes of this microorganism may contaminate the intestines of animals, water and foods in general. The list of the most susceptible foods to contamination by *Salmonella* spp. is long, and includes meats in general, eggs, milk and dairy products, fish, some kinds of sweets, and others (CDC, 2013a; Foley et al., 2008; Fortuna et al., 2012).

The National Program of Pathogen Reduction, Microbiological Monitoring and *Salmonella* spp. Control in Chicken and Turkey Carcasses was published in Ordinance 70, 2003, to establish standards and quality control measures for poultry products, and developed an information system on the pathogen, guaranteeing food safety for domestic and export markets (Brasil, 2003). The Brazilian legislation established the threshold of zero bacterial count of *Salmonella* spp. in 25 g of a bird meat sample (Brasil, 2001).

Contamination by *Salmonella* spp. is stressed in food poisoning outbreaks. In the USA, approximately 9.4 million cases of food-borne diseases are reported a year. Laboratory analyses confirmed that 19,531 infections were associated with food-borne pathogens in the country in 2012, and *Salmonella* spp. were implicated in 16.42% of the confirmed food poisoning cases (CDC, 2013b; CDC 2013c).

In Brazil, between 2000 and 2011, of the 3,927 food poisoning cases reported, 1,660 were due to *Salmonella* spp. Concerning the origin of infection, 51.84% of these cases were acquired at home, and 17.93% in restaurants and similar places. The foods most commonly implicated in these poisoning cases were prepared with a mixture of ingredients, with eggs, as well as sweets and desserts, water, *in natura* beef, processed meat and offal (Secretaria de Vigilância em Saúde, 2011).

Contamination of foods by *Salmonella* spp. is due to inappropriate handling and hygiene conditions, among other reasons. The person preparing foods plays a crucial role in contamination, since the hand is one of the main vehicles of microorganism transmission (CDC, 2013d; Fortuna et al., 2012). In a study on the microbiological quality of hands of 44 workers in 13 state schools in Brazil, 2.3% were positive for *Salmonella* spp. (Souza and Santos, 2009). After the analysis of food, eggs, mayonnaise and chicken implicated in 10 salmonellosis outbreaks the researchers suggested that the possible causes of contamination were mistakes in handling, which promoted cross-contamination. A study on a salmonellosis outbreak in a restaurant chain revealed that raw chicken, seasoning and the chopping board used to slice cooked chicken were responsible for contamination of foods by *Salmonella* serotype Montevideo (Patel et al., 2010).

The clinical characteristics of salmonellosis are diarrhea, nausea, abdominal pain, mild fever, shivers, and occasional vomiting, headaches and weakness. In some cases, people affected may present more severe signs. During the disease, the patient releases the bacterium via feces, which may be spread in the environment (CDC, 2013d).

The processing of foods in restaurants has to obey safety standards. Chan and Chan (2008) discovered that restaurants were the most common sources of food-borne etiological agents between 1996 and 2005. In a study that investigated the presence of *Salmonella* spp. in feces of workers who prepared food in two university canteens, four (10%) were positive for the bacterium, which represents a serious public health problem (Sandrea Toledo et al., 2011). Also, in another study, the presence of *Salmonella* spp. was detected in salads in over 50% of commercial restaurants surveyed in the city of Rio de Janeiro, Brazil. Problems concerning cross-contamination, poor personal hygiene of restaurant workers and in the control in food storage were considered critical (Antonio and Ghisi, 2011). Several studies carried out in kitchens and restaurants point to problems in constructive, physical, functional, hygienic and sanitary aspects both in preparation of foods and in physical installations and utensils, in the training of workers and personal hygiene, which are critical points in the production of safe foods (Bramorski et al., 2008; Colombo et al., 2009; Faheina Jr et al., 2008).

The objective of the study was to detect the presence of *Salmonella* spp. in chicken meat *in natura* and ready for consumption, in the hands of employees, in personal protective equipment and in utensils used and in the food preparation process, considering food safety in accordance with official regulations. This information may be useful in the development of more efficacious food preparation procedures.

MATERIALS AND METHODS

Epidemiological study

An exploratory-descriptive and quantitative study, including data survey and laboratory investigation was carried out. Data were collected by systematic observation using an instrument to record the work process throughout the production chain of foods, from the moment when raw foods are received to the distribution of ready food in the restaurant. In this survey, the facts and phenomena studied were known and examined (Lakatos and Marconi, 2011).

The laboratory investigation included the bacteriological analyses

to detect *Salmonella* spp. according to the American Public Health Association (APHA) (Andrews et al., 2001). Analyses were carried out in the Laboratory of Microbiological Control of Animal Products, Department of Food Technology, Veterinary Medicine College, Universidade Federal Fluminense (UFF).

Samples were collected in an industrial kitchen that prepares meals in a university restaurant, in the city of Niteroi, State of Rio de Janeiro, Brazil. The kitchen produces approximately 6,000 meals a day, 4,200 at lunch and 1,800 at dinner, consumed by students and employees in general of the University, in site and in other restaurant units of the University. Dinner is offered in the main unit and in a secondary unit.

The university kitchen was chosen because it is considered a large production center of meals for the academic community, to improve students' performance and because the kitchen does not have a microbiological control program for the meals it serves.

The investigation was carried out between April 2012 and January 2013, and was divided in two phases. The first phase included the collection of all samples, with no interference to researcher. The second took place after the results obtained in the first stage were presented to the manager and the employees of the kitchen, with the subsequent implementation of improvements in the work process in light of the results obtained during the first phase.

The meetings to present the results of the analyses were carried out using the quality tools of the plan, do, check, action (PDCA) cycle, the Cause and Effect Diagram and the Technical Regulation of Best Practices in Production. At the end of this process, new operational methods were described directed to the safe production of foods in the kitchen.

Bacteriological analyses

Sample collection

Thirty-six chicken meat samples were randomly collected (18 in each stage) in the kitchen: three samples during preparation and three samples during distribution and consumption. The chicken cuts sampled were: deboned chicken breast (DCB), deboned skinless whole leg (DSL), and whole leg (with bones and skin, WL).

Collection of raw chicken samples was carried out during preparation. This stage of the processing of chicken included the thawing, the rinsing in running water and vinegar, and the addition of a seasoning mix prepared with vinegar, salt, garlic, onion and bay leaves. Collection of finished food took place during the distribution of meals, in a university restaurant that receives the transported meals. The raw and cooked food samples were from the same batch of meals distributed in the menu. There, three containers with chicken food are delivered daily. One sample was retrieved from each container.

In stages 1 and , the deboned chicken breast was minced and cooked in a steam cooker for 60 min on average, while the whole leg (with bone and skin) and the deboned and skinned whole leg were prepared in a combined oven for 55 min, on average.

Bacteriological analyses were carried out using 250 g samples of chicken meat (for both raw and cooked chicken), as recommended in the official literature (Brasil, 2001). Samples were randomly collected and placed in sterile polyethylene bags. All samples were transferred to the laboratory immediately after collection in a thermal plastic reusable bag containing ice. Collection of samples for the bacteriological analyses followed the procedures outlined by LACEN (2010).

Besides, and during the two stages of the study, six samples were collected from hands and of personal protective equipment (four rubber gloves, two from mail gloves, two from silicon gloves, six from aprons and two from masks) of the employees working in preparation, cooking and distribution areas, were collected using sterile swabs, totaling 22 samples. Workers were randomly selected. Personal protective equipment is individually used and is compulsory, for the safety and health of workers (Brasil, 2010). Additionally, samples were collected from utensils (two from the plastic chopping boards, two from containers and respective covers, two from stainless steel spatulas) as specified by APHA (Andrews et al., 2001), totaling eight samples, in the two stages of this study. These utensils were chosen because they are used in the preparation and distribution of chicken. These swab samples were collected during the food processing activities, with no previous hygiene, except for the samples collected from the hands of preparation workers. The samples collected from the containers and lids were obtained before they were used. The analysis of hands, personal protective equipment and kitchen utensils and followed the same methodology, but samples were collected using a disposable sterile swab as described by APHA (Andrews et al., 2011) LACEN (2010).

Collection of all samples, at the different stages of meat production, was preceded by the observation of procedures at the different stages of meal production, according to a predefined program of routine inspection and description (Lakatos and Marconi, 2011). The chicken meat cuts were randomly retrieved throughout the production process. During observation of the procedures, temperatures of chicken meat lots used for sample collection were measured, at the different steps, using a specific meat thermometer (Incoterme™).

In the laboratory, a 25 g portion was obtained from each chicken sample using sterile instruments and homogenized in a stomacher with 225 ml buffered peptone saline 1%. Then, the mixture was incubated at 37ºC for 24 h. Then, two 1 ml aliquots were retrieved and seeded in two separate tubes, one containing 10 ml Rappaport Vassiliadis (RV) broth (Himedia M880-500 g) and one containing 10 mL EE Mossel (M) broth (Himedia M287-500 g). The tubes were then incubated at 37 and 41ºC, respectively, for 24 h.

Each suspension was then seeded on disposable sterile Petri dishes containing the following selective media: Hektoen (H) Agar (Himedia M467-500 g), Brilliant Green Agar Base (BPLS) (MicroMED 2164), and *Salmonella* Differential Agar (SS) (Himedia M1078-500g) and the dishes were incubated upside down for 18-24 h at 36°C.

The dishes presenting typical *Salmonella* spp. colonies were picked, and five colonies obtained (from each culture medium) were streaked on Triple Sugar Iron (TSI) agar (Himedia M021-500 g) and immediately incubated 36ºC for 24 h at 36°C. Typical *Salmonella* spp. growths were selected and an inoculum was obtained from the center of the dish using a needle and seeded on separate tubes containing Phenylalanine agar (Himedia M281-500 g) and Nutrient agar (Himedia M090-500 g), and incubated for 24 h at 35°C. Then, 3 to 5 drops of ferric chloride 10% were added to the tubes containing the Phenylalanine agar. Sterile saline was added to the positive *Salmonella* spp. cultures the Nutrient agar and one drop of the suspension was retrieved and transferred to a glass slide for serological test to ascertain the self-aglutination capacity of the isolate. One drop of *Salmonella* Polyvalent serum (Probac do Brasil™) was then added and confirmation of a positive result was obtained by agglutination of serum in contact with the suspension analyzed.

The analyses of hand, personal protective equipment and utensils followed the same methodology. The only difference was the initial dilution of these samples, which was carried out in a test tube containing 90 mL buffered peptone saline 1%, and homogenized. Samples were diluted by adding buffered peptone saline 1% and homogenize.

Statistical analyses

The data obtained were analyzed by the Student's t test to assess

Table 1. Number and percentage of positive samples for *Salmonella* spp. in each chicken cut, in preparation and distribution, in each of the stages.

Cut	Preparation				Distribution			
	Stage 1		Stage 2		Stage 1		Stage 2	
	N	%	N	%	N	%	N	%
Deboned chicken breast (DCB)	1	33	2	67	2	67	0	-
Deboned skinless whole leg (DSL)	1	33	0	-	0	-	0	-
Whole leg (WL)	3	100	2	67	1	33	1	33
Mean (%)	55.6 %		44.5%		33.4 %		11.2 %	

significant differences between mean results independently for each sample type (meat and surfaces). Statistical significance was $\alpha = 0.05$.

Ethics approval

This research, which is part of a PhD dissertation, was approved by the Ethics Committee of the Medicine College, Teaching Hospital Antônio Pedro, Universidade Federal Fluminense (protocol CAAE 0417.0.258.000-11).

RESULTS

The results are presented for the two stages including those for chicken meat samples (biological analyses) and hands, personal protective equipment, kitchen utensils and equipment (surfaces). Of the 18 chicken meat samples analyzed in the first stage, eight (44.4%) were positive for *Salmonella* spp. in the chicken meat cuts analyzed, except the deboned skinless whole leg (DSL) in distribution, which were negative for *Salmonella* spp. (Table 1).

In the first stage, the samples presenting the highest number of positive results were whole leg (with bone and skin, WL), with three positive samples (100%). In distribution, the highest percentage of isolation occurred in DCB (deboned chicken breast), with two (67%) of positive samples (Table 1).

In the second stage, of the 18 samples analyzed, in preparation and in distribution, five (27.8%) were positive for *Salmonella* spp. (Table 1). The samples with the highest prevalence of positive results were deboned chicken breast (DCB), with two (67%) positive samples, and whole leg (WL), with two (67%) positive samples, in preparation. In distribution, only one (33%) sample of whole leg (WL) was positive for *Salmonella* spp. (Table 1).

A reduction in microbial load was observed in food, between the stages; however, the Student's t test showed that there were no significant differences between mean results in the two stages, in spite of the changes implemented in the operational process, both in preparation and distribution, with significance level $\alpha = 0.05$.

In the analysis of hands, protective equipment and kitchen utensils, the following results were obtained: in stage 1, in the preparation area, *Salmonella* spp. were isolated from hands, apron and mail gloves; in the cooking area, the bacteria were isolated in the rubber gloves a and b; in the distribution area, it was isolated in masks, totaling six (40.0%) positive samples of samples (Table 2).

In stage 2, *Salmonella* spp. were isolated in the preparation area, in the following samples: hands, rubber gloves, apron, mail glove and chopping board. In the cooking area, the bacteria were isolated in aprons. In the distribution area, it was isolated in hands a and b. In total, eight (53.33%) of samples were positive (Table 2).

A difference was observed in microbial loads between hands, personal protective equipment and utensils, in the two stages. However, the Student's t test revealed no significant differences between mean results of the two stages (1 and 2) with significance level $\alpha = 0.05$.

The results of the means of the temperatures measured in the preparations that presented positive *Salmonella* results are presented in Table 3. These data are relevant for the comprehension of the process of cross-contamination in terms of the operational flow.

In stages 1 and 2 the deboned chicken breast was minced and cooked in a steam cooker for 60 min on average, while the whole leg (with bone and skin) and the deboned and skinned whole leg were prepared in a combined oven for 55 minutes, on average. Between stages 1 and 2, meetings were held with the manager, nutritionists and workers to analyze and discuss the results of the microbiological analyses and observations carried out, followed by the presentation of new work methodologies. At the end, several suggestions were carried out, to improve quality of the techniques used and consequently of the final product. The PDCA cycle was used to define objectives and a new systematization of the procedures (Figure 1). The cause and effect diagram was essential to identify and analyze problems and propose suggestions.

Table 4 presents the mistakes observed and the solutions implemented. The mistakes observed and for which no solutions were implemented are listed at the end of the table. The results of the observational analysis

Table 2. Number of positive samples positive for *Salmonella* spp. In hands, personal protective equipment and utensils in preparation and distribution, in each of the stages.

Section	Surface	Stage 1	Stage 2	Sections
Preparation	Hand	02	positive	positive
	Apron	02	positive	positive
	Rubber glove	02	absence	positive
	Mail glove	02	positive	positive
	Chopping board	02	absence	positive
Cooking	Rubber glove (a)	01	positive	No personal equipment in this stage
	Rubber glove (b)	01	positive	No personal equipment in this stage
	Silicon glove	01	No personal equipment in this stage	absence
	Silicon glove	01	No personal equipment in this stage	absence
	Apron	02	absence	positive
	Stainless steel container	02	absence	absence
	Cover	02	absence	absence
	Stainless steel spatula	02	absence	absence
Distribution	Hand (a)	02	absence	positive
	Hand (b)	02	absence	positive
	Apron	02	absence	absence
	Mask	02	positive	absence
Mean (%)			40%	53.3 %

Table 3. Mean temperatures of prepared and cooked chicken cuts, with positive results for *Salmonella* spp. in the distinct stages of the production cycle.

Cut	Stage 1			Stage 2		
	Preparation (°C)	Main kitchen (°C)	Distribution (°C)	Preparation (°C)	Main kitchen (°C)	Distribution (°C)
DCB	10.6	82.8	63.4	9.5	81.0	63.4
WL	5.9	85.5	64.6	8.34	87.8	70
DSL	8.1	93.5	75.7	13.2	90.8	76.5

DCB: Deboned skinless chicken breast, DSL: Deboned skinless whole leg (in combined oven), WL: Whole leg

4 Act: Definition of new proposals in terms of monitoring, with a view to continuous improvement	**1 Plan:** Assessment of the operational model used and implementation of the required changes; meetings with the workers involved
3 Check: The proposals implemented are evaluated systematically? The evaluation of results is carried out?	**2 Do:** Meetings with workers; data analysis, discussions and proposals; implementation of proposals

Figure 1. Analysis of the production process in the area of meat preparation based on the PDCA cycle.

Table 4. Problems identified in the observational analysis with implications for the microbiological analyses, and solutions proposed by managers and kitchen workers.

Area	Problems identified	Solutions proposed and implemented
PREPARATION	Mistakes in operational flow	Establishment of new operational flows to prevent crossings and backflows
PREPARATION	Problems in physical-functional structure	Works to correct physical problems
COOKING	Use of rubber gloves in cooking	This item will not be allowed in this area; only disposable gloves will be used
COOKING	Inappropriate personal protective equipment and utensils	Purchase of silicon gloves and of specific utensils for the area
COOKING	Mistakes in operational flow	Establishment of new operational flows to prevent crossings and backflows
PREPARATION/ COOKING/ DISTRIBUTION	Mistakes in hand hygiene	Training on the correct procedure
PREPARATION/ COOKING/ DISTRIBUTION	Mistakes in procedures of hygiene and sanitation of the physical area, utensils and equipment	Definition of a standardized operational program for hygiene and sanitation. Purchase of appropriate equipment and hygiene and sanitation products
PREPARATION/ COOKING/ DISTRIBUTION	Circulation of people who do not work in the area	The circulation of people who do not work in the area should be restricted
PREPARATION/ COOKING/ DISTRIBUTION	Problems in the division of utensils for the areas, such as knives, containers, etc.	Purchase and identification of utensils for each area
	Problems that were identified but were not solved	**No correction**
PREPARATION/COOKING	Mistakes in conformity with physico-structural structure	
PREPARATION	Absence of a walk-in refrigerator for the thawing of meat and for prepared meat Structural problems in the existing walk-in refrigerators	
PREPARATION/ COOKING/ DISTRIBUTION	Mistakes in the supervision of people along the production chain and in the detailing of responsibilities	
PREPARATION/COOKING/ DISTRIBUTION	Mistakes in the systematic microbiological control of foods and surfaces	
PREPARATION/COOKING/ DISTRIBUTION	Deficiency in the technical training of kitchen workers	

were not quantified, only described in a report, for the two stages.

DISCUSSION

In the present study, in food preparation, *Salmonella* spp. was isolated during both stage 1 and stage 2. In stage 1, results were positive for deboned chicken breast (DCB), deboned skinless whole leg (DSL), and whole leg (with bones and skin, WL). In stage 2, positive results were observed for deboned chicken breast (DCB), deboned skinless whole leg (DSL). This may indicate cross-contamination during any stage of the chicken production

chain, but it may also be due to contamination by spices, since the samples analyzed at this stage were already seasoned. However, it should be remembered that *Salmonella* spp. is not significantly implicated in the contamination of spices *in natura.*

Contamination of chicken meat by *Salmonella* spp. may indicate hygienic and sanitary issues in breeding sites, during slaughter or during handling of animals thereafter, as reported by several authors. In fact, Duarte et al. (2009), in a study that analyzed 260 chicken carcasses from five different processing plants, identified *Salmonella* spp. in 9.6% of tested samples. Contamination with *Salmonella* spp. was also detected by Borsoi et al. (2010), in a study that analyzed 180 chilled chicken carcasses, the pathogen was identified in 12.2% of samples. However, Nierop et al. (2005) report a higher value, 19.25% of 99 chicken carcasses were contaminated by *Salmonella* spp.

Duarte et al. (2009) and Nde et al. (2007), underline the fact that there is *Salmonella* spp. contamination in chicken breeding sites and flocks, and warn of the subsequent introduction of the bacterium the slaughter houses. In fact, the pathogen present in feathers and skin may contaminate the meat during slaughter, raising the bacterial counts on structures, equipment, utensils and even in scalding water, in a cross-contamination process that extends throughout the production chain. The endresult of such unsafe process is food with an inappropriate degree of microbiological quality, which represents a health hazard for the end consumer.

Franco (2012), underline that the investigation of *Salmonella* spp. is very important, since the bacterium plays a crucial role in the epidemiology of food poisonings due to its more severe etiopathogeny. For this reason, the analytical sampling is more representative (25 g), with acceptable threshold defined as the absence of the pathogen in this sample, while the threshold established for other microorganisms is given based on 1 g of the food matrix.

Indu et al. (2006), Pereira et al. (2006), and Castanha et al. (2010) investigated the antibacterial action of extracts as potential inhibitors of pathogenic agents, even of *Salmonella* spp. However, Fuselli et al. (2004) also pointed to the problems with–microbiological quality in terms of contamination by bacteria commonly observed in the environment, such as spores, fungi, some micrococci and mycobacteria.

It should be emphasized that hands, personal protective equipment used in the preparation of food were positive for *Salmonella* spp. (Table 3). This result may be associated with contamination of chicken meat with the bacterium, lending strength to the occurrence of cross-contamination in utensils and workers, and vice-versa. Nde et al. (2007) investigated the cross-contamination by *Salmonella* spp. in an abattoir and reported the event when hygienic and sanitary controls are ineffective. Malatova et al. (2009), in a study carried out in a commercial restaurant, identified the presence of *Salmonella* spp. in the hands of kitchen staff after processing foods that were positive for the pathogen. In a set of recommenddations on the prevention of *Salmonella* spp. Contamination, CDC (2013d) underlines the importance of the care taken with surfaces involved in food preparation.

In the industrial kitchen investigated in the present study, during the observation of the activities in the preparation area, both in stages 1 and 2, even after the implementation of new procedures, mistakes were identified concerning hand sanitation, use of protective equipment well as sanitation of the work area and in the management procedures in general. It was observed that preparation staff constantly circulated in other areas of the food production chain, with no due care to cleaning hands or removing personal protective equipment.

In the preparation area, workers hygienized their hands before collection, but positive results were observed for samples in the two stages (Table 2), which indicates the inadequacy of the process. A similar result was obtained by Gonçalves et al. (2013), in a study on the microbiological quality of hands of food preparation workers after hygienization, where the authors detected the presence of enteropathogenic bacteria.

In a study on the hygiene of hands, Cruz et al. (2009) indicate, as the likely causes for low adherence to the practice, the role of behavior, habits, the environment and deliberate intention. The managers of the kitchen carry out yearly training on several themes associated with safe food preparation. However, mistakes in hand hygiene persist, which may indicate issues concerning the adherence to this practice, since the facilities are equipped with the physical installations for the purpose.

Regarding the work methods in the kitchen investigated in the present study, in the preparation area, chicken meat was thawed in a chamber with no refrigeration, or in the area where meat is going to be prepared, the air conditioner on and set at 18ºC. After the chicken meat was removed from the package, it was left there for between 5 and 6 h on average, until seasoned and stored at 4ºC.

Franco (2012) reported that time and temperature control is essential to prevent the growth of *Salmonella* spp., since it does not thrive successfully in temperatures under 5ºC and above 47ºC. The samples analyzed in the present study were not kept under temperatures considered safe for preparation of chicken meat (Table 4), raising microbiological contamination concerns.

Considering that several studies address the occurrence of *Salmonella* spp. in chicken meat, it can be inferred that, in the event of an initial contamination the bacterium may develop where inappropriate time-temperature conditions associated with other factors like environment, equipment, hand hygiene and cross-contamination, prevalent.

The objective of the present study was to investigate the presence of *Salmonella* spp. during the different stages

of the processing of the food matrix, and to identify hygienic and sanitary conditions as of its consumption, but not to trace the microorganism presence from the farming unit and, transportation to the industrial processing plant. Therefore, it is not possible to ascertain that the positive results for *Salmonella* spp. reported here are due to problems happening in the farm or during transportation, since the equipment and utensils employed in the production chain and the workers may have carried the bacterium. Yet, Duarte et al. (2009) and Nde et al. (2007) described that poultry may be asymptomatic carriers of the pathogen.

Regarding the chicken prepared for consumption, positive results for *Salmonella* spp. were observed in three samples (DCB and WL) in stage 1 and in one sample in stage 2 (WL) (Table 2). These results configure the inadequacy of technical procedures of handling of foods after cooking, since during this process the foods were exposed to temperatures and times considered high and long enough to eliminate the bacterium (Table 4). According to Franco (2012), the minimum temperature to reduce viable cells (in a 6 log scale) is 70ºC for 2 min in a humid environment. However, according to a recommendation by ICMSF (1996), *Salmonella* spp. may be eliminated at 60ºC for 15 to 60 min, since these microorganisms are non-sporulated and are thermoliable.

The observation of processes during sample collection affords to conclude that; (i) the time between preparation of chicken in the kitchen and distribution was 4 h; (ii) to be transported, the prepared food is placed in a stainless steel container, which is placed in a hot box, (iii) the hot box is open only at the moment of distribution.

It is possible to conjecture that the contamination of the food ready for consumption takes place because of the cross-flow of the product, promoted by staff, from the preparation, the cooking a and distribution areas, due to problems in the handling of the food after cooking. This fact is confirmed by the positive results for *Salmonella* spp. in the hands and personal protective equipment of workers in the cooking and distribution areas, both in stages 1 and 2 (Table 2), even after the implementation of the new work procedures (Table 4). Another aspect that reinforces this hypothesis is the fact that the temperature reached by the cooked chicken may ensure the elimination of the pathogen.

The problems observed may have linked direct relationship with the microbiological results obtained in this study. In stage 1, one worker handled chicken ready for consumption wearing a reusable rubber glove, circulating in the kitchen; after they were removed from the ovens, trays were placed on top of one another, containing the food and with no protection in between; the preparation staff circulated in the cooking area wearing the same personal protective equipment; the raw chicken meat was kept near the cooked chicken in the cooking area; the gloves worn to handle raw chicken and those worn to handle the product ready for consumption were placed side by side, in an inappropriate place.

Concerning the circulation of staff, it should be stressed that this was a recurring habit in the kitchen, even after the implementation of the new procedures, defined between the two stages of this research. It was noticed that no instruction or restriction is in place for this matter: people circulate between the different work areas wearing the same protective equipment. This behavior promotes cross-contamination, since it carries microbial loads between work spaces.

After training, technical procedures were corrected. However, old, inappropriate habits in the handling of foods reemerged, with positive results for the etiological agent investigated, in food, in hands and on surfaces in stage 2. Concerning the food, possible to observe that, during the collection of samples, of DCB and DSL, in distribution, in stage 2, it was workers followed the new procedures strictly. However, in the WL samples, old mistakes were made, like touching the cooked food with the hands and piling up trays after removing them from ovens with no physical protection in between.

The results obtained in the present study were similar to the findings by Chan and Chan (2008), in a study carried out in Japan, between 1996 and 2005. The authors reported the occurrence of 5,967 outbreaks of food poisonings, of which 6.47% were due to poor hygiene of workers, and restaurants were the main sources of these infections.

A similar result was obtained by Guimarães et al. (2001), who investigated the microbiological quality of ready meals and the implications concerning the people handling these foods. The authors detected *Salmonella* spp. in beans and sautéed cassava. *Salmonella* Typhy, *Salmonella* Enteritidis and *Salmonella* spp. strains were isolated from staff handling these foods. In like manner, Madalosso et al. (2008) in a similar study in a commercial restaurant, associated the cause of the outbreak of food poisoning by *Salmonella* Enteritidis with the blender used in food preparation.

Considering cross-contamination as an important factor in the maintenance of *Salmonella* in food raw materials, Rubin et al. (2012) isolated *Salmonella* spp. from the hands of staff in charge of preparing food, from different surfaces, from a cloth used to tap-dry surfaces and from a sponge in an area used for food production.

Opposite results to those obtained in the present study were reported by Mesquita et al. (2006), who analyzed the microbiological quality of roast chicken in a food unit and obtained positive *Salmonella* spp. results in roast chicken, in hands, in containers or in counters. The same analytical profile was assessed by Vasconcelos and Filho (2010) in a study about the microbiological quality of meals prepared in the kitchens of commercial restaurants.

However, CDC (2013d) recommends the prevention to the contamination by *Salmonella* spp. based on the correct hand hygiene practices during preparation and

cooking procedures and during the handling of different food items, the hygiene of surfaces after contact with raw foods, mainly meat, and the avoidance of the contact of raw foods with cooked dishes, preventing cross-contamination.

In the present study, a decrease in the studied pathogen counts was observed after the training program and the implementation of procedural changes (Table 1). Changes were made in the operational flow, in the processing of chicken meat, in hygiene and sanitation procedures, in time and temperature control, and in the handling of cooked chicken, to improve service quality (Table 4). Nevertheless, this is not an acceptable result, since in Brazil the microbiological threshold is zero *Salmonella* spp. counts in 25 g of cooked meat and in the present study the pathogenic agent was isolated in the WL samples and in hands and personal protective equipment (Table 2), after the changes implemented. I can be concluded that the mistakes in the management of the service and in the handling of food remain.

It should be said that control measures have to be in place to monitor food processing, starting at the purchase of raw material of better quality and ending at the distribution of the food prepared, so as to reduce risks to the nutritional, sensory, physical, chemical and microbiological quality of cooked meals. It is only through these measures that it is possible to guarantee the production of safe foods.

Conclusion

The high number of samples positive for *Salmonella* spp. *in natura* chicken meat, in the meat ready for consumption and on the surfaces analyzed in the present study showed that the procedures adopted in preparation and cooking are inappropriate.

Salmonella spp. is a fecal bacterium, and therefore it is essential to control the quality of the raw material used, the preparation procedures, and the hygienic practices of staff in working areas, utensils and personal protective equipment. Services presenting these kinds of problems should review or implement control protocols and redefine procedures and techniques to minimize and correct these issues.

It is only with the adoption of a mindset that aims to protect the worker and the end consumer that it will be possible to meet the requirements defined according to the right to healthy, quality nourishment.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Characterization of nematode community in apple orchard soil in the Northwestern Loess Plateau, China

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This work was carried out in the Northwestern Loess Plateau (NLP), one of two main large-scale apple production areas in China. It is to investigate the role of nematodes in apple production areas, which cause apple replant disease (ARD). Soil samples from total eight sites in NLP were collected twice, in summer and autumn, respectively. The nematodes were extracted by washing-sifting-sucrose centrifugation and total nematodes in each sample were counted with the aid of an anatomical lens (40×). Furthermore, a sub-sample of one quarter of each nematode suspension was observed with a Motic microscope (400 and 1000×) and each nematode was identified to genus level using diagnostic keys. The results indicated that the characteristics of the nematode community did not vary significantly in four different types of soils. Especially, the diversity indices of nematode community did not show obvious differences. In addition, the analysis of nematodes with different feeding habits revealed that the overall number of *Pratylenchus penetrans* nematodes in the replanted orchard in NLP were far below the density threshold that could jeopardize apple trees. Thus, it appeared that the nematodes were not the leading causal agent for ARD in NLP.

Key words: Apple replants disease, nematode diversity, nematode feeding habits, nematode community structure.

INTRODUCTION

The Northwestern Loess Plateau (NLP) is a large-scale apple cultivation area, however, most orchards are old and while production is decreasing, they need to be replaced, which makes growers face apple replant disease (ARD). ARD is a soil-borne disease that often occurs when trees are replanted in soils which have history of apple growth. It has been reported from all major fruit-growing regions of the world (Mazzola and Manici, 2012), and ARD is often caused by a consortium of biological agents, mainly, including nematodes, bacteria, actinomycete, oomycetes and fungi species (Tewoldemedhin et al., 2011). Nematode is an important soil organism. Nematodes occur widely in various types of soil, with numerous species and great mass, and they

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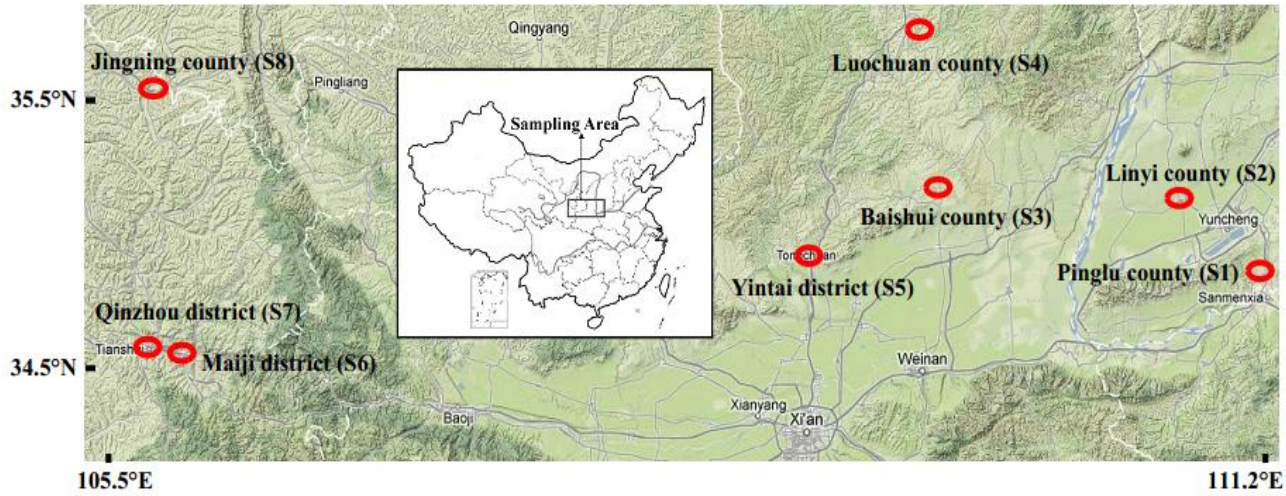

Figure 1. Map of eight sampling locations (red ellipses) of apple orchards in the Northwestern Loess Plateau, China.

play an important role in the soil ecological process by changing the food webs structure and decomposition approach, affecting the functions of soil ecosystems (Ferris et al., 2001; Yeates and Bongers, 1999). ARD is a soil-borne disease, and thus, a soil environment approach should be taken to explore the pathogenesis of the disease. A good soil environment is the premise for the healthy growth of fruit trees. Foreign ecological scholars have proposed that soil nematode communities have been widely used as bioindicators of ecosystem conditions (Yeates, 2003; Ritz et al., 2009; Sánchez-Moreno et al., 2010), due to their key positions in soil food webs (Neher, 2001). The utilization of nematode community analysis for indicating soil food web dynamics in agroecosystems has been reported by many researches (Ferris and Matute, 2003; Briar et al., 2007; Sánchez-Moreno et al., 2006; DuPont et al., 2009).

Ecological indices calculated from relative populations of different soil nematode groups have been widely applied to quantify the response of the nematode community to environmental changes in soil (van Eekeren et al., 2008; Neher et al., 2005; Todd et al., 2006). The ShannoneWeaver Index (*H*) is a useful measure of diversity of nematode community (Cheng et al., 2008; Zhang et al., 2009). Indices of free living nematode maturity (MI) and plant-parasitic nematode maturity (PPI) represent soil nematode life-history characteristics associated with r-selection (nematode with short generation time, high fecundity and large population fluctuation) and K-selection (nematodes with long generation time, few offspring and generally appearing later in succession), respectively (Neher, 2001). MI and PPI have often been used to estimate the functional responses of soil nematodes to environmental change (Chen et al., 2009; Yeates and Newton, 2009).

Given the above, we carried out the project in apple region; which will be discussed here. The objectives of our study were to determine the differences of four cropping histories on soil nematode abundance and diversity in NLP, and to explore the role of soil nematode in ARD.

MATERIALS AND METHODS

Study site outline

The study was carried out in NLP area, which is the world's largest loess deposition area. The plateau is slightly north of central China (34-40°N, 103–114°E). The climate belongs to semi-humid region; the mean annual temperature here is about 8–14°C; annual precipitation varies from about 600-800 mm.

Soil sampling

Individual soil samples were collected in summer (June 3 to 9) and autumn (September 28 to October 6) of 2010 at eight sampling sites (each site being a replicate). The sites were located in Pinglu (S1) and Linyi (S2) counties of Shanxi province; Baishui (S3), Luochuan (S4) counties and Yintai (S5) district of Shaanxi province; Maiji (S6), Qinzhou (S7) districts and Jingning (S8) county of Gansu province, respectively (Figure 1 and Table 1).

At each sampling site, soils with the following four cropping histories were sampled: 1) replanted apple orchards; 2) within rows of old apple orchards; 3) between rows of old apple orchards (=inter-row) and 4) fallow soil. The replanted apple orchards had been replanted five years ago and suffered from ARD. The old apple orchards had been planted twenty years ago. In both cases, soil was sampled within a radius of about 0.5 m around the taproot of the tree. The fallow soil was located close to the apple orchard, but had not previously been planted with apple trees (= soil bare). At each point, two replicate samples were taken to a depth of 20 cm. Thus, a total of 128 soil samples were collected (2 seasons × 8 sites × 4 cropping histories × 2 replicates). All soil samples were sifted through a 6 mm sieve to remove the plant roots or large stones, subsequently placed in black plastic bags and stored at

Table 1. Soil characteristics and climate of eight sampling sites of the NLP. Data represent mean ± standard error of 8 replications. Within each column, values followed by different low case letters are significantly different according to one-way ANOVA and Duncan's new multiple range test ($P \leq 0.05$).

Sampling site	Latitude/longitude	Average annual rainfall (mm)	Soil classification	Average annual temperatures (°C)
S1	E111.2° N34.12°	600-700	Cinnamon soil	13.8
S2	E110.78° N35.15°	500-800	Cinnamon soil	13.5
S3	E109.6° N 35.18°	578	Loessial soil	11.4
S4	E109.42° N35.76°	620	Dark loessial soil	9.2
S5	E109.11°N35.09°	539-740	Loessial soil	11.0
S6	E105.7° N34.58°	506-754	Cinnamon soil	10.0
S7	E105.89° N34.57°	531	Loessial soil	10.7
S8	E105.73° N35.51°	451	Loessial soil	7.1

Pinglu (S1) and Linyi (S2) of Shanxi (SX) province; Baishui (S3), Luochuan (S4) and Yintai (S5) of Shaanxi (SN) province; Maiji (S6), Qinzhou (S7) and Jingning (S8) of Gansu (GS) province.

4°C. Each sample was extracted and counted separately and the values of the two replicate samples were averaged.

Nematode extraction and identification

Nematodes were extracted from 100 g wet soil by washing-sifting-sucrose centrifugation, using sieves with 40 (350 µm) and 500 (25 µm) meshes. The nematodes were heat-killed at 60°C and preserved in triethanolamine formaldehyde (TAF) solution (Shepherd, 1970). Total number of nematodes in each sample were counted with the aid of an anatomical lens (40×); a sub-sample of one quarter of each nematode suspension was observed using an inverted compound microscope (Motic; 400 and 1000x). All nematodes in the samples were identified, to genus level if possible. The nematodes were assigned to the following trophic groups: (1) bacteriovores (BF); (2) fungivores (FF); (3) plant-parasites (PP); and (4) omnivores-predators (OP) (Yeates et al., 1993). C-p values were allocated according to Yeates and Bongers (1999) and ecological indices were calculated as shown in Table 2. The abundances of total nematodes and each taxonomic group were adjusted to the number of soil nematodes per 100 g dry soil.

Statistical analysis

The effect of cropping history on the ecological indices was analyzed separately for each season with one-way ANOVA using DPS V7.05, which was a statistical analysis software developed by Zhejiang university of China, function equated with SPSS. Differences among treatment means were analyzed with Duncan's multiple range test.

RESULTS

Richness and density of nematode

The effect of cropping history on nematode richness (number of genera) and density was significant difference both in summer and in autumn. In summer, nematode richness in fallow soil was the highest of the four cropping histories (Table 3). In contrast, the lowest nematode richness was found in the vicinity of apple trees in both replanted and old orchards. The richness in inter-row soil was intermediate and not significantly different from the other soils. Accordingly, nematode density in fallow soil was significantly higher than that in the other soils.

In autumn, nematode richness was significantly higher in replanted orchards and fallow soil than in the old orchards (Table 3). Nematode density was significantly higher in fallow soil than in the old orchards. Nematode density was intermediate in replanted orchards, but not significantly different from the other soils.

Nematode community structure

The effect of cropping history on Shannon-Wiener diversity (*H'*), Simpson diversity (*D*), Pielou evenness (*J*) and Margalef richness (*SR*) was significant both in summer and in autumn. In summer, the value of the indices did not significantly differ among the four cropping histories (Tables 4 and 5). In autumn, *J* and *SR* did not significantly differ among the four cropping histories. However, in autumn *H'* was significantly higher in replanted than in old orchards and *D* was significantly higher in replanted orchards than within rows in old orchards.

Functional group features

Among the soils, no significant differences were found in Structure Index (SI), Enrichment Index (EI), Maturity Index (MI), Plant-parasite Index (PPI) and PPI/MI (Tables 6 and 7). SI index and EI index in summer presented a similar variation. Namely, gradually decreased as follows: replanted soil, row soil, inter-row soil and fallow soil. Nevertheless, there were no significant differences among them. SI index and EI index in autumn did not vary in a consistent manner, but there were also no

Table 2. Formulae used to calculate community indices for soil nematodes. Data represent mean ± standard error of 8 replications. Within each column, values followed by different low case letters are significantly different according to one-way ANOVA and Duncan's new multiple range test ($P \leq 0.05$).

Index	Formula	Interpretation	References
Shannon-Wiener diversity *(H')*	$H' = -\sum_{i=1}^{n} pi * \ln pi$	*p* is the proportion of individuals in the ith taxon A measure of taxonomic diversity An increase in the index indicates increasing diversity	Shannon and Weaver,1949
Simpson diversity *(D)*	$D = 1 - \sum_{i=1}^{n} p_i^{\,2}$	*p* is the proportion of individuals in the ith taxon	Simpson,1949
Pielou evenness *(J)*	$J = H' / \ln S$	S is the number of taxa H' is the Shannon-Weiner diversity	Pielou,1969
Margalef richness (*SR*)	$SR = (S-1) / \ln N$	S is the number of taxa N the number of individuals identified	Yeates and Newton, 2009
Structural index (*SI*)	$SI = 100 * (s/(s+b))$	s is the abundance of those guilds indicative of an increasingly structured food web b is the abundance of those bacterial and fungal feeding guilds indicative of the basal food web condition Ranges from 0-100 Increasing SI indicates increased environmental stability	Ferris et al., 2001
Enrichment index (*EI*)	$EI = 100 * (e/(e+b))$	e is the abundance of those bacterial and fungal feeding guilds indicative of resource enrichment b is the abundance of those bacterial and fungal feeding guilds indicative of the basal food web condition Ranges from 0-100 Increasing EI indicates increased resource availability	Ferris et al., 2001
Maturity index (*MI*)	$MI(\sum MI) = \sum_{i=1}^{n} (v(i) * f(i))$	v(i) is the c-p value of the ith taxon, and f(i) is the frequency of the ith taxon	Bongers,1990 Yeates,1994
Plant-parasite index (*PPI*)	$PPI = \sum_{i=1}^{n} (v(i) * f(i))$	v(i) is the c-p value of the ith taxon, and f'(i) is the frequency of the ith taxon	Bongers,1990

obvious differences among themselves, respectively. Although MI index, PPI index and PPI/MI values for four cropping histories soils in summer slightly varied, the statistical significance was not obvious. Of course, PPI index was higher than MI index. The statistic results of values in autumn were analogous to those in summer.

Nematode feeding habits

The effect of cropping history on the number of BF, FF, PP, OP nematodes and *Pratylenchus penetrans* was significant both in summer and in autumn. In summer, no significant differences were found in the number of BF, PP and OP nematodes among the four cropping histories (Table 8). In summer, the number of FF nematodes and *P. penetrans* was higher in fallow soil than in replanted apple orchards and between rows in old apple orchards. However, in autumn the number of FF nematodes did not significantly differ among the four cropping histories. In contrast, in autumn the number of BF, PP, OP nematodes and *P. penetrans* did significantly differ among the cropping histories (Table 8). The number of BF was significantly higher in replanted orchards than in the other soils. The number of PP nematodes and *P. penetrans* was significantly higher in fallow soil than in the old orchards, whereas the number of OP was significantly higher in fallow soil than in all three other soils.

DISCUSSION

Soil nematodes have been widely used as bioindicators to assess soil condition as they respond to changes in the soil environment caused by land use, agricultural management, etc. (Bongers and Ferris, 1999; Dong et al., 2008; Liang et al., 2009; Neher et al., 2005). Ecological indices have been widely applied to explore

Table 3. Richness (number of genera) and density (number per 100 g dry soil) in summer and autumn of soil with four different cropping histories: replanted apple orchards, old apple orchards (within rows and inter-rows) and fallow soil (n=8). Data represent mean ± standard error of 8 replications. Within each column, values followed by different low case letters are significantly different according to one-way ANOVA and Duncan's new multiple range test ($P \leq 0.05$).

Treatment	Richness		Density	
	Summer	Autumn	Summer	Autumn
Replant soil	11.13±1.61b	13.00±0.98a	127.40±29.79b	139.48±17.91ab
Row soil	10.63±0.63b	9.50±0.76b	136.41±39.28b	70.80±15.48b
Inter-row soil	12.88±1.29ab	10.13±0.67b	150.20±47.61b	97.25±18.36b
Fallow soil	15.38±1.36a	13.75±0.92a	302.16±40.66a	215.69±65.48a

Table 4. Shannon-Wiener diversity (*H'*) and Simpson diversity (*D*) of nematodes extracted from soil with four different cropping histories in summer and autumn. Data represent mean ± standard error of 8 replications. Within each column, values followed by different low case letters are significantly different according to one-way ANOVA and Duncan's new multiple range test ($P \leq 0.05$).

Treatment	*H'*		*D*	
	Summer	Autumn	Summer	Autumn
Replant soil	1.87±0.22a	2.27±0.10a	0.73±0.07a	0.86±0.02a
Row soil	1.79±0.21a	1.82±0.15c	0.74±0.08a	0.77±0.05b
Inter-row soil	2.02±0.09a	1.97±0.04bc	0.81±0.02a	0.82±0.01ab
Fallow soil	1.97±0.06a	2.15±0.03ab	0.78±0.02a	0.85±0.01ab

Table 5. Pielou evenness (*J*) and Margalef richness (*SR*) of nematodes extracted from soil with four different cropping histories in summer and autumn. Data represent mean ± standard error of 8 replications. Within each column, values followed by different low case letters are significantly different according to one-way ANOVA and Duncan's new multiple range test ($P \leq 0.05$).

Treatment	*J*		*SR*	
	Summer	Autumn	Summer	Autumn
Replant soil	0.75±0.05a	0.89±0.02a	1.94±0.28a	2.50±0.18a
Row soil	0.76±0.08a	0.81±0.06a	2.11±0.17a	2.06±0.20a
Inter-row soil	0.83±0.03a	0.85±0.01a	2.28±0.24a	2.13±0.08a
Fallow soil	0.74±0.03a	0.83±0.02a	2.52±0.20a	2.49±0.08a

the response of nematode community to soil environmental changes (Todd et al., 2006; van Eekeren et al., 2008), the soil nematode community can be used as a biological marker of soil health (Wardle et al., 1995; Neher, 2001; Neher et al., 2012; Klass et al., 2012), and evaluate soil quality (Yeates and Wardle, 1996; Yeates and Bongers, 1999).

The ecological indices of *H'*, *SR* and *MI* are often used to assess the soil condition. *H'* and *SR* are linked to the diversity of soil nematodes, and *MI* can reflect changes in soil condition (Zhang et al., 2009). In addition, *SI* and *EI* parameters can also be an indicator for soil health (Ferris et al., 2001). They were the most frequently used indices in the field of soil nematode community providing ecology in recent years (Powell, 2007). The present results showed that ecological indices, e.g. H', D, J, SR, SI, EI, MI, PPI and PPI/MI, except H' and D varied in four cropping histories soils in summer, but no evident differences were found by statistical significance in summer and autumn. Based on the above fact and result, we concluded that there were no differences among four cropping histories soils in soil nematode community structure, and there is a stable soil ecosystem. However, with regards to nematode, it did not demonstrate that four cropping histories soils have the same soil condition and soil health only from the above ecological indices, the whole factors should be considered, not one by one.

Many studies suggest that nematodes are useful

Table 6. Structural index (*SI*) and enrichment index (*EI*) of nematodes extracted from soil with four different cropping histories in summer and autumn. Data represent mean ± standard error of 8 replications. Within each column, values followed by different low case letters are significantly different according to one-way ANOVA and Duncan's new multiple range test ($P \leq 0.05$).

Treatment	*SI*		*EI*	
	Summer	Autumn	Summer	Autumn
Replant soil	65.13±9.13a	49.18±5.47a	48.59±4.92a	31.46±2.83a
Row soil	51.73±9.07a	46.15±7.56a	48.02±7.23a	40.26±7.73a
Inter-row soil	48.51±7.94a	47.23±7.84a	43.06±8.33a	38.49±7.09a
Fallow soil	47.21±7.97a	59.37±5.42a	41.24±7.87a	28.93±3.56a

Table 7. Maturity index (*MI*), plant-parasite index (*PPI*) and PPI/MI of nematodes extracted from soil with four different cropping histories in summer and autumn. Data represent mean ± standard error of 8 replications. Within each column, values followed by different low case letters are significantly different according to one-way ANOVA and Duncan's new multiple range test ($P \leq 0.05$).

Treatment	*MI*		*PPI*		*PPI/MI*	
	Summer	Autumn	Summer	Autumn	Summer	Autumn
Replant soil	1.02±0.14a	1.57±0.21a	1.27±0.28a	0.89±0.11a	1.29±0.25a	0.70±0.19a
Row soil	0.99±0.11a	1.22±0.19a	1.18±0.22a	0.98±0.24a	1.40±0.35a	1.23±0.49a
Inter-row soil	1.16±0.17a	1.37±0.22a	1.18±0.20a	0.96±0.19a	1.41±0.47a	1.09±0.40a
Fallow soil	1.00±0.07a	1.46±0.18a	1.04±0.17a	1.25±0.18a	1.10±0.25a	0.98±0.30a

Table 8. Number of different feeding habits soil nematodes and *P. penetrans* extracted from soil with four different cropping histories in summer and autumn. Data represent mean ± standard error of 8 replications. Within each column, values followed by different low case letters are significantly different according to one-way ANOVA and Duncan's new multiple range test ($P \leq 0.05$).

Treatment	BF		FF		PP		OP		*Pratylenchus penetrans*	
	Summer	Autumn	Summer	Autumn	Summer	Autumn	Summer	Autumn	Summer	Autumn
Replant soil	39±11a	59±11a	5±1b	10±2a	69±19a	52±10ab	15±6a	20±4b	34±2c	19±2c
Row soil	34±8a	32±10b	13±4ab	3±1a	61±19a	34±11b	12±3a	7±2b	24±1d	11±2d
Inter-row soil	35±8a	33±5b	6±2b	5±2a	86±36a	45±15b	23±6a	14±4b	46±1b	29±1b
Fallow soil	39±8a	22±5b	24±6a	10±4a	121±15a	107±33a	20±4a	38±11a	62±2a	46±1a

bioindicators in soil and aquatic ecosystems, especially for assessment of ecosystem health (Ferris and Bongers, 2006; Shao et al., 2008; Neher, 2010; Park et al., 2011). PP nematode is well-known pests affecting important agricultural crops such as apple, peach, cotton and soybeans, and is one of the causes of continuous cropping obstacles crop. On this account, some researchers thought that *Meloidogyne incognita*, *P. penetrans* and *Heterodera glycines* are the main pathogenic nematodes in continuous cropping obstacles crop (St Laurent et al., 2008; Sharma and Kashyap, 2004; Stetina et al., 2007; Koenning and Edmisten, 2008). Hoestra and Oostenbrink (1962) showed that high densities of PP nematodes played an important role in ARD, primarily *Pratylenchus* spp. *P. penetrans* Cobb is considered to be the primary nematode species involved in ARD (Van Schoor et al., 2009; Tewoldemedhin et al., 2011). Jaffee et al. (1982) described that *P. penetrans* was the most abundant parasitic nematode of PP nematodes, once its counts was up to 140 *P. penetrans* per 100 cm^3 and it would result in significant stunting and root necrosis. Results of the number of different nematode feeding habits in NLP apple region showed that the largest number of PP nematodes appeared in fallow soil, which was 121 per 100 g dry soil, and the largest number of *P. penetrans* appeared in fallow soil which was 62 per 100g dry soil, and was below the density threshold that could jeopardize apple trees. Thus, it appeared that the nematode was not the leading causal agent for apple replant disease in NLP.

Conclusion

Nematode populations in soils subjected to different agricultural practices have shed additional light on the effects of tillage systems and environmental conditions,

including temperature, moisture and soil compaction. This paper discussed the soil nematode community characteristic of replant soil, row soil and inter-row soil of old orchards as well as fallow soil in NLP apple region without taking into account the above factors and other microorganism effect, which need to be further studied. Thus, the variety in orchard soil nematode community performance could reflect the change in orchard soil environment quality. The understanding could provide a basis for the old orchard's update or alteration and prevention of apple replant disease.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Bio-detoxification of *Jatropha curcas* seed cake by *Pleurotus ostreatus*

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The detoxification of *Jatropha curcas* seed cake is of major interest for the biodiesel industry to add economic value to this residue and also to reduce the environmental damage caused by its inappropriate disposal. In this context, the treatment of this residue with white rot fungus, *Pleurotus ostreatus,* can be a viable alternative because it produces enzymes capable of degrading different lignocellulosic residues and toxic compounds. In this study, the capacity of *P. ostreatus* to degrade phorbol esters found in *Jatropha* seed cake and the potential to transform this residue in animal feed was evaluated. After 60 days of incubation with the fungus, the phorbol ester concentration was reduced by 99% (final concentration of only 2 x 10^{-4} mg g^{-1} dry mass). This value is lower than the level observed in the non-toxic Mexican variety. Also, we showed that fungal growth improved some features desirable for animal feed, such as, increases the *in vitro* digestibility, decreases lignin and cellulose content and increases the protein content. Therefore, *P. ostreatus* is able to degrade phorbol esters found in *Jatropha* seed cake and has the potential to be used as animal feed.

Key words: Physic nut, toxic compounds, phorbol ester, biodiesel, lignocellulosic residue.

INTRODUCTION

In Brazil, the National Program for Production and Use of Biodiesel published in 2004 inserted the biofuel in the national energy matrix and its production became an alternative to energy self-sufficiency.

Biodiesel is produced from vegetable oils and fats by a transesterification reaction with mono- or dialcohol (Openshaw, 2000). Due to its drought hardiness, easy propagation, low cost of seeds, high oil content and wide adaptation, *Jatropha curcas* has been widely used for biodiesel production (Sujatha et al., 2008). After extracting oil from *J. curcas*, a solid residue, called seed cake, is produced (Openshaw, 2000; Patil and Deng, 2009). This seed cake is rich in lignocelluloses and proteins (Makkar et al., 1997) and has great potential to be used as a biofertilizer, for biogas production or as animal feed (Gubitz et al., 1999). However, toxic compounds, such as phorbol esters and curcin, and antinutritional factors, such as tannins and phytic acid, make this residue

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improper for direct animal consumption and cause environmental damage if disposed of inappropriately (Goel et al., 2007; Makkar et al., 1997). Curcin and the antinutritional factors can be degraded by thermal or biological treatments (Aregheore et al., 2003; Kasuya et al., 2012; Rehman and Shah, 2005). da Luz et al. (2013) showed the degradation of antinutricional factors by *Pleurotus ostreatus*.

The phorbol esters found in the seed and oil were identified as the main toxic agents of *J. curcas* (Makkar et al., 1997; Sudheer et al., 2009; Salimon and Ahmed., 2009). Animal consumption of these phorbol esters can cause diarrhea and inflammation of the gastrointestinal tract and death (Makkar and Becker, 1997). The most varieties of *J. curcas* cultivated in world are toxic, only one non-toxic variety is found in Mexico (Makkar et al., 1997; Pamidimarri et al., 2009). Thus, detoxification of *Jatropha* seed cake is necessary for their use as animal feed.

White rot fungi (*P. ostreatus, Trametes versicolor, Phanerochaete chrysosporium, Pycnoporus sanguineus*) are able to grow and degrade various pollutants and recalcitrant compounds, such as polycyclic aromatic hydrocarbons (Majcherczyk et al., 1998), tinitrotolueno (Nyanhongo et al., 2006) and phytate (Kasuya et al., 2012) by the action of several lignocellulolytic enzymes, for example laccase, manganese peroxidase (MnP), cellulase and xylanase (Barr et al., 1994; Honggiang and Hongzhang, 2008; Lamarino et al., 2009; Majcherczyk et al., 1998). The above assumptions show that these fungi probably can degrade toxic compounds (e.g. phorbol esters) found in *Jatropha* seed cake. This detoxification would allow the use of seed cake for the production of enzymes and edible mushrooms and as animal feed. Indeed, it has been shown that intake of Jatropha seed cake colonized by *P. ostreatus* is not toxic to goats (Kasuya et al., 2012). This detoxification would allow the use of seed cake for the production of enzymes and edible mushrooms and as animal feed. Thus, the aim of this study was to evaluate the capacity of *P. ostreatus* to degrade phorbol esters found in *Jatropha* seed cake.

MATERIALS AND METHODS

Microorganism, fungal growth conditions and inoculum production (spawn)

The isolate Plo 6 of *P. ostreatus* (GenBank accession number KC782771, 2013) was grown in a Petri dish containing potato dextrose agar culture medium (Merck, Darmstadt, Germany), at pH 5.8, and incubated at 25°C. After seven days, the mycelium was used for inoculum production (spawn) in a substrate made of rice grains. The rice was cooked for 30 min in water at a 1:3 ratio of rice: water (w/w). After cooking, the rice was drained and supplemented with 0.35% $CaCO_3$ and 0.01% $CaSO_4$. This rice (70 g) was packed into small glass jars and sterilized in an autoclave at 121°C for 1 h. After cooling, each jar was inoculated with 4 agar discs (5 mm diameter) containing mycelium and incubated in the dark at room temperature for 15 days.

Substrates used and inoculation

To select the most suitable substrates for lignocellulolytic enzyme production, preliminary experiments were conducted with *Jatropha* seed cake and different lignocellulosic residues. *P. ostreatus* was grown in *Jatropha* seed cake with different percentages of eucalypt sawdust, corn cob, coffee husk and eucalypt husk. The addition of these agroindustrial residues was designed to balance the carbon and nitrogen relationship, which might benefit mycelial growth (Elisashvili et al., 2008; Giardina et al., 2000; Shashirekha et al., 2005).

Based on the results of these preliminary experiments, the compositions selected for biological detoxification are as shown in Table 1. The substrates were humidified with water at 75% of retention capacity, and 1.5 kg of each substrate was placed in polypropylene bags and was autoclaved at 121°C for 2 h. After cooling, the substrates were inoculated with 75 g of spawn and incubated at 25°C. Samples of non-inoculated bags were also kept.

Enzymatic assays

After 15, 30, 45 and 60 days of incubation, 10 g of each substrate was placed in Erlenmeyer flasks (125 mL) containing 25 mL of sodium citrate buffer (50 mM, pH 4.8). The flasks were kept in a shaker for 30 min at 150 rpm, and extracts were filtered with Millipore membranes (Cavallazzi et al., 2004). Enzyme assays were performed in triplicate, and enzyme activity was calculated as the difference in absorbance between non-inoculated and inoculated samples.

Laccase and manganese peroxidase (MnP) activities were measured using 2,2'-azino-bis-3-etilbenzotiazol-6-sulfonic acid (Buswell et al., 1995) and phenol red solution (Kuwahara et al., 1984) as substrates, respectively. Xylanase and cellulase activities were calculated by measuring the levels of reducing sugars that were produced by the enzymatic reactions (Bailey et al., 1992; Mandels et al., 1976).

One unit of enzyme activity was defined as the amount of enzyme required to catalyze the production of one µmol of colored product or reducing sugars per mL per min.

Chemical composition of the substrates

Lignin, cellulose and hemicellulose content were determined as described by Hatfield et al. (1994).

Tannins and phytic acid were quantified by a colorimetric method as described by Makkar et al. (1995) and Gao et al. (2007), respectively.

The manganese, copper and zinc content found in *Jatropha* seed cake that was not inoculated, were evaluated by plasma emission spectrometry (Perkin Elmer, M Optima 3300 DV optical Inductively Coupled Plasma Emission Spectrometer) after acid digestion.

The level of reducing sugars was determined by the DNS method (99.5% dinitrosalicylic acid, 0.4% phenol and 0.14% sodium metabisulfite) and the standard curve was made with D-glucose (Merck, Darmstadt, Germany) with the concentrations of the standard ranging from 0.5 to 1.5 g L^{-1}.

Phorbol ester determination

Phorbol ester content was analyzed by high performance liquid

Table 1. Substrate compositions used for *P. ostreatus* growth.

Substrate	Mass substrates (kg)	
	J. curcas	Agroindustrial residue
Jatropha seed cake (Jc)	20	0
Jc + 10% eucalypt sawdust (JcEs)	18	2
Jc + 10% eucalypt husk (JcEh)	18	2

chromatography (HPLC), as described by Makkar et al. (1997). Three grams of the substrate were triturated for 10 min in a porcelain mortar. These samples were transferred to centrifuge tubes (50 mL) containing 20 mL of methanol (Sigma) and centrifuged at 4000 x*g* for 10 min at 4°C. The supernatant was filtered on paper filter (Whatman GF/D, degree 2.5 cm). An additional 10 mL of methanol was added to the solid material retained in the membranes, which was again centrifuged and filtrated. The supernatant of the first and second filtrations were transferred to Erlenmeyer flasks (125 mL), dried in a vacuum (40°C) in rotavapor (Büchi, 461 Water Bath) and resuspended in 5 mL of tetrahydrofuran (Sigma). Twenty microliters of this suspension was injected into the HPLC (Shimadzu, C18 reverse phase and UV detection at 280 nm) and eluted with a gradient of acetonitrile and 0.175% orthophosphoric acid (Makkar et al., 1997). The standard curve was generated using phorbol-12-myristate 13-acetate (Sigma) with concentrations ranging from 0.005 to 0.5 mg mL^{-1}. The retention time of the standard was between 41 and 52 min, with four characteristic phorbol ester peaks (Makkar et al., 1997).

Biomass determination

The fungal biomass was determined by ergosterol content according to Richardson and Logendra (1997). Five grams of substrate colonized by fungus was triturated for 10 min in a porcelain mortar containing 0.3 g polyvinylpyrrolidone (Sigma) and 15 mL ethanol 95%. Then, this material was centrifuged during 20 min at 4.200 *g* and 4°C. The supernatant was filtered on Teflon sieve (200 mm x 53 µm) and stored at 4°C. Twenty microliters of this suspension was injected into the HPLC (Shimadzu, CLC-ODS reverse phase and UV detection at 280 nm) and eluted with methanol (Sigma) at 1.0 mL min^{-1}. Standard curve was prepared with ergostatrien-3β-ol (Sigma) dissolved in ethanol 95%.

Dry mass of the substrates

To determinate the dry mass, 1.5 kg of the substrate was dried at 105°C until a constant weight was obtained (Kasuya et al., 2012; Silva and Queiroz, 2002).

Chemical composition of Jatropha seed cake

To determine the dry mass (DM), organic matter (OM), crude protein (CP) and mineral matter (MM) we used the methodology describe by Silva and Queiroz (2002). The content of non-fibre carbo-hydrates (NFC), lignin, hemicellulose and cellulose were evaluated as describe by Van Soest et al. (1991). Ether extract (EE) was evaluated as describe by the American Oil Chemistry Society (AOCS, Official procedure Am 5-04, Ankon technology). The crude protein was measured with the Bradford method (Bradford, 1976).

In vitro digestibility

To measure the dry matter *in vitro* digestibility, we used the method describe by Tilley and Terry (1963) with minor modifications. We collected ruminal liquid of fistulated cattle from the department of animal husbandry (Universidade Federal de Viçosa), about two hours after feeding. The samples were incubated at 39°C for 30 min for anaerobic bacteria selection. We incubated 350 mg of each samples in anaerobic jars with continuous flow of CO_2 and a solution containing 4 mL ruminal liquid and 32 mL of McDougall buffer. The jars were incubated at 39°C for 48 h at 120 rpm. After the incubation, we filtered the samples in porcelain crucibles and washed with hot water until complete removal of the McDougall buffer. Then, we added 70 mL of neutral detergent solution and autoclaved at 121°C for 15 min. After this, the samples were washed again with hot water until complete removal of neutral detergent solution and washed once with 10 mL of acetone. The crucibles were maintained in 105°C for 16 h. The samples were put in the desiccator and their masses were measured.

Statistical analysis

The experiment was a completely randomized design with 5 replicates. The data were subjected to analysis of variance and mean values were compared by Tukey's test ($p < 0.05$) using Saeg software (version 9.1, Universidade Federal de Viçosa).

RESULTS AND DISCUSSION

The chemical composition of the substrates was significantly altered after heat treatment and, generally, there was a decrease in lignin, cellulose, antinutritional factor and phorbol ester content (Table 2). Several authors have shown a decrease in lignocellulosic compounds and antinutritional factors after heat treatment (Alvarez and Vázquez, 2004; Makkar et al., 1997; Rehman and Shah, 2005).

Dry mass, biomass and lignocellulosic compounds degradation

The dry mass loss (Figure 1A) correlated positively with the percentage of lignin degradation (significance = 0.154) (Figure 2A) and negatively with biomass (significance = 0.354) (Figure 1B), as also reported by Berg (2000). Furthermore, in the degradation of *Quercus petraea* leaves by the basidiomycetes, *Hypholoma fasciculare*

Table 2. Compounds found in substrate used for *P. ostreatus* growth.

Compound	Substrate (mg g^{-1})					
	Thermal treatment					
	Before			After		
	Jc	JcEs	JcEh	Jc	JcEs	JcEh
Lignin		See Figure 2			See Figure 2	
Cellulose		See Figure 3			See Figure 3	
Monosaccharides	8.25 ± 1.14	7.51 ± 0.11	6.30 ± 0.67	15.88 ± 5.64	14.96 ± 3.16	19.91 ± 1.49
Tannin*	2.50 ± 0.12	0.95 ± 0.44	3.01 ± 0.70	1.24 ± 0.34	0.56 ± 0.09	1.49 ± 0.11
Phytic acid	3.08 ± 0.28	2.31 ± 0.02	2.33 ± 0.17	1.08 ± 0.007	0.77 ± 0.009	0.77 ± 0.001
Phorbol ester	1.07 ± 0.02	0.63 ± 0.01	0.72 ± 0.01	0.73 ± 0.01	0.47 ± 0.01	0.56 ± 0.001
Manganese	0.044	nd	nd	nd	nd	nd
Copper	0.022	nd	nd	nd	nd	nd
Zinc	0.036	nd	nd	nd	nd	nd

* Contents are reported as equivalents of tannic acid; nd- not determined; Jc- *Jatropha* seed cake, JcEs – *Jatropha* seed cake + 10% eucalypt sawdust, JcEh10- *Jatropha* seed cake + 10% eucalypt husk (see Table 1).

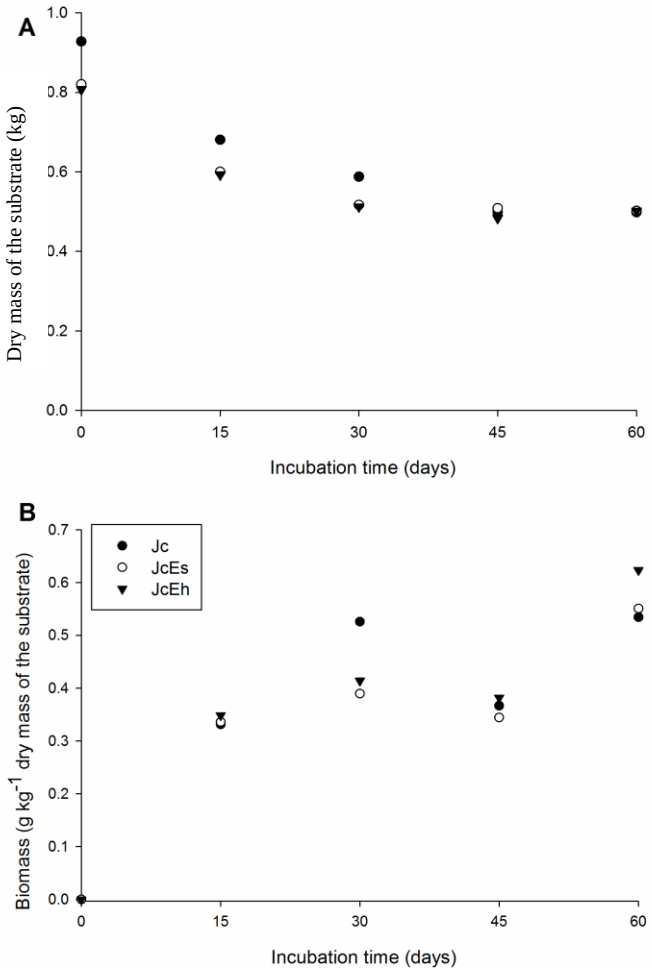

Figure 1. Dry mass (A) and mycelial biomass production (B) during 60 d of incubation with *P. ostreatus* in substrates with different proportions of *Jatropha* seed cake.

and *Rhodocollybia butyracea*, similar correlations were observed between dry mass and lignocellulolytic enzyme activity (Valáskova et al., 2007).

The reduction of total dry mass was, on average, 46% in seed cake and 39% in the other substrates (Figure 1A). However, these total losses dry mass were lower than that obtained by *P. ostreatus* in wheat straw after 50 days of incubation (Baldrian and Gabriel, 2003) and in lingocellulosic residues after 98 days (Baldrian and Gabriel, 2002). According to those authors, the high loss of dry mass was due to presence of copper, manganese and zinc in the substrate that stimulated the activity of the lignocellulolytic enzymes (Cavallazi et al., 2004). In this context, the high laccase and MnP activities (Figure 2B and C), which resulted in high lignin degradation (Figure 2A), can also be explained by the availability of these element in *Jatropha* seed cake (Table 2). The presence of copper in the laccase binding center stimulates the activity of this enzyme (Koroleva et al., 2001). MnP is a glycoprotein that has iron as prosthetic group and dependent on the hydrogen peroxide and manganese concentration for lignin oxidation (Brown et al., 1991; Hofrichter, 2002).

The high lignin degradation (Figure 2A), associated with greater dry mass loss (Figure 1A) and high ligninase activity (Figure 2B and C), suggesting that this polymer was the main source of carbon used for biomass production in Jatropha seed cake and in the substrate containing eucalypt sawdust (Figure 1B). This result could be due to a higher initial lignin concentration than cellulose and monosaccharide in these substrates (Table 2).

The higher cellulose degradation (Figure 3A) and also higher xylanase activity (Figure 3B) were observed in substrate containing eucalypt husk. The fungal biomass production in these substrates was due to, mainly, high

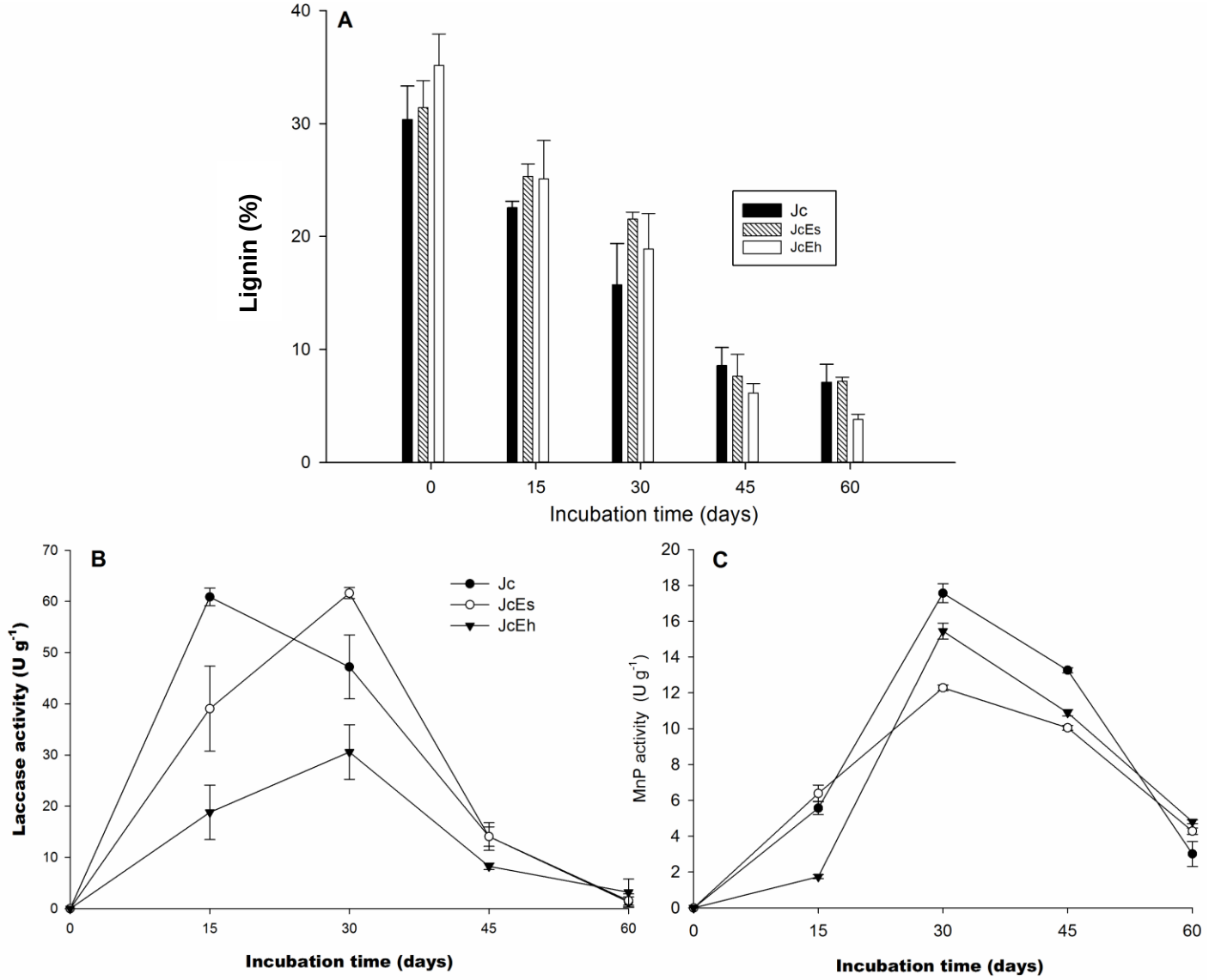

Figure 2. Lignin percentage (A) and laccase (B) and manganese peroxidase activity (C), during 60 days of incubation with *Pleurotus ostreatus* in substrates with different proportions of *Jatropha* seed cake.

monosaccharide availability (Table 2, Figure 1B) and xylanase activity (Figure 3B), because cellulase activity (Figure 3C) and laccase activity (Figure 2B) were low. This high monosaccharide availability (Table 2) may have influenced the differential expression of lignocellulolytic enzymes. This differential expression and also the role of these enzymes in the enzymatic degradation complex of lignocellulosic compounds have also been shown in *L. edodes* and *P. ostreatus* cultivated in different substrates (Cavallazi et al., 2004; Elisashvili et al., 2008).

High cellulase activity in Jatropha seed cake and substrate containing eucalypt sawdust shows a synergistic effect of this enzyme with ligninases (Figure 2 and 3C). Therefore, the use of lignin as a source of carbon and energy requires substrates containing cellulose or other carbon sources, as suggested by Ruggeri and Sassi (2003). It is important to observe that there was fungal biomass production (Figure 1B) and enzymatic activity (Figure 2 and 3) in Jatropha seed cake without addition of none lignocellulosic residues. With these results we reached the following conclusions: a) for the mycelial growth and fungal enzyme production, there is no need for the addition of lignocellulosic residues and b) the phorbol ester and antinutritional factors concentration found in Jatropha seed cake was not toxic for *P. ostreatus* (Table 2). Thus, this fungus can be used to

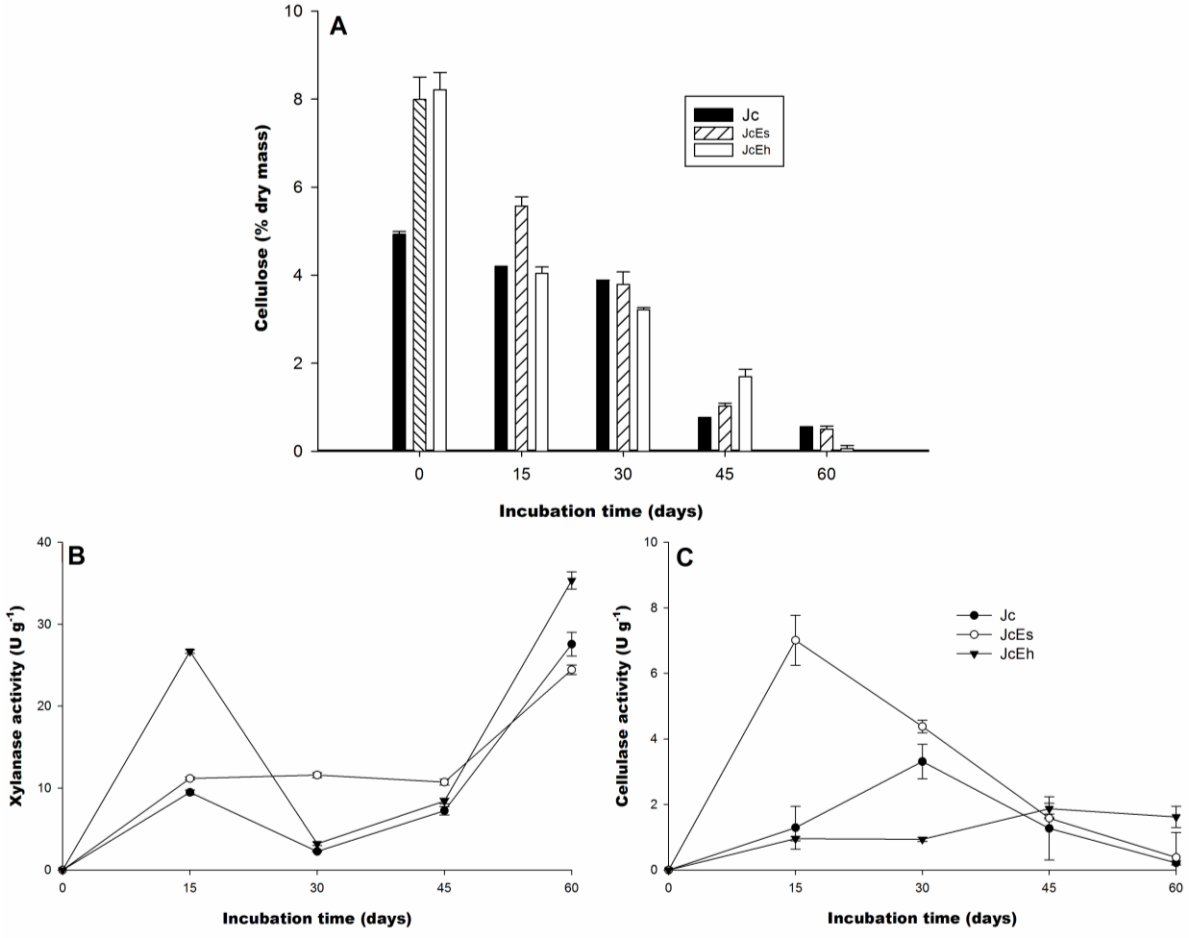

Figure 3. Cellulose percentage (A) and xylanase (B) and cellulase activity (C), during 60 days of incubation with *Pleurotus ostreatus* in substrates with different proportions of *Jatropha* seed cake.

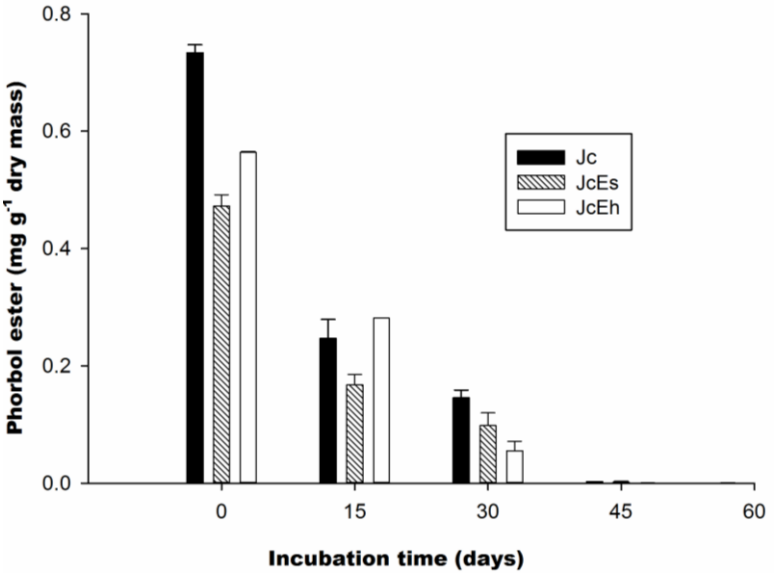

Figure 4. Phorbol ester degradation for *Pleurotus ostreatus* in substrates with different proportions of *Jatropha* seed cake.

Table 3. Chemical composition (%) of *Jatropha* seed cake added with agro-industrial residues colonized or not by *P. ostreatus* for 45 days.

Components	Jc		JcEs		JcEb	
	non-inoculated	colonized	non-inoculated	colonized	non-inoculated	colonized
Dry mass (DM)	95.03	96.24[a]	94.83	96.31[a]	95.03	96.08[a]
Organic matter (OM)	93.30	91.14[a]	93.97	92.49[a]	92.74	91.97[a]
Crude protein (CP)	11.44	13.16[a]	10.74	12.15[a]	11.08	11.26[a]
Ether extract (EE)	17.93	7.56[a]	16.82	7.08[a]	16.21	7.10[a]
Non-fibre carbohydrates (NFC)	63.94	70.92[b]	66.42	73.26[a]	65.26	73.80[a]
Mineral matter (MM)	6.70	8.86b	6.03	7.51[b]	7.26	8.03[b]
In vitro digestibility dry mass	54.90[b]	77.92	60.06[a]	80.44	60.31[a]	83.90

Jatropha curcas seed cake (Jc), Jc + 10% of eucalyptus sawdust (JcEs), Jc + 10% of eucalyptus bark (JcEb); See Table 1. Means followed by the same letter in the same line, do not differ by analysis of variance and Tukey test (P<0.05).

degrade lignocellulosic compounds of the Jatropha seed cake (Figure 2A and 3A) and to increase the organic matter and non-fibre carbohydrate content and digestibility of this residue as observed by Kasuya et al. (2012), using different percentages of Jatropha seed cake biodetoxified in the ration of goats.

Phorbol ester degradation

The initial phorbol concentration found in *Jatropha* seed cake was between 0.63 and 1.07 mg g^{-1} (Table 2). This concentration was higher than that found in the non-toxic variety from Mexico (Makkar et al., 1997) and lower than that found in varieties of Cape Verde and Nicaragua (Makkar and Becker, 1997; Martínez-Herrera et al., 2006). Therefore, these results demonstrate that phorbol concentration in the *J. curcas* seed can be influenced by the growing region and variety.

Autoclaving the substrates at 121°C reduced, on average, 20% the phorbol ester content (Table 2). However, these compounds were not degraded by treatment at 160°C for 30 min (Aregheore et al., 2003). Moreover, the addition of sodium hydroxide and sodium hypochlorite combined with heat treatment was able to reduce the phorbol concentration by 25% (Goel et al., 2007). In this work, *P. ostreatus* was able to degrade 99% of the phorbol ester content after 60 days of incubation (Figure 4). This rate of degradation was higher than that obtained by chemical deodorization, deacidification, or bleaching agents applied to the oil and seed cake of *J. curcas* (Rakshit et al., 2008). The same chemical processes, with the exception of bleaching, when applied to *J. curcas* seed, were not effective in reducing phorbol esters (Salimon and Ahmed, 2009).

The observed phorbol ester degradation (Figure 4) may be explained by the capacity of *P. ostreatus* to deploymerize lignin. As observed by Barr and Aust (1994) and Majcherczyk et al. (1998), the degradation of organic compounds, for example chlorophenols and aromatic hydrocarbons, occurs due to the lignin depolymerization reaction, mainly through the activities of laccase and MnP. The activities of these enzymes from *Phanerochaete* sp. (Perez et al., 1998) and *P. ostreatus* (Lamarino et al., 2009) have also been reported in the decolorization of various dyes in the textile industry and in the elimination of different pollutant compounds.

Maximum phorbol ester elimination did not occur until the 30th incubation day (Figure 4) and coincided with the interval of high laccase and MnP activity (Figures 2B and 2C). However, other enzymes could have influenced this degradation because the degradation of phenols and furfural by cellulase has been observed by Honggiang and Hongzhang (2008). In our study, higher cellulase and xylanase activities (Figure 3) were observed between the 15th and 45th incubation days as indicated by 37 and 60% of phorbol ester degradation, respectively. In the substrate containing eucalypt husk, however, lower phorbol ester degradation and lower ligninases activity were observed on the 15th day of incubation (Figures 2B, C and 4). This evidence supports the hypothesis that phorbol ester degradation occurs by co-metabolism with the enzymes responsible for lignin depolymerization.

Finally, after 60 days of incubation with *P. ostreatus*, the residual phorbol ester concentration was, on average, 2 x 10^{-4} mg g^{-1} dry mass (Figure 4). This concentration is much lower than the 0.09 mg g^{-1} found in the non-toxic Mexican variety of *Jatropha* (Aregheore et al., 2003). Furthermore, Kasuya et al. (2012) observed no clinical symptoms of poisoning in goats fed with *Jatropha* seed cake detoxified by *P. ostreatus* and concluded that biodetoxified *Jatropha* seed cake can be safely used in the goat diet.

Chemical composition of *Jatropha* seed cake

The colonization of some agro-industrial residues by microorganism has been successfully used as strategy to increase their digestibility and nutritional value. The growth

of *P. ostreatus* in *Jatropha* seed cake added or not with agroindustrial residues improved some features desirable to animal feed. We observed an increase in CP, NFC, MM and a decrease in EE, lignin, cellulose, hemicellulose contents (Table 3). Our data is similar to the chemical composition of other goat feeds (Lee et al., 2008; Khan et al., 2009).

The CP is the main source of essential amino acids to the animals (NRC, 2000; Verbic, 2002). This is one of the principal factors used to assess the potential of agro-industrial residues to be used in animal feed. Therefore, the increase in CP due to fungal growth is beneficial to ruminants (Table 3).

The decreases in EE content (Table 3) contributes to the use of *Jatropha* seed cake in animal feed, because is recommended for diet dry matter with less than 10% of EE (Mohammed et al., 2004). According to Palmquist and Mattos (2011), lipid content higher than 5% of dry mass consumption is due to (a) regulatory mechanisms which control food intake or (b) the limited ability of ruminants to oxidize fatty acids.

The fungal growth improves the *in vitro* digestibility of all substrates tested (Table 3). This may be related to the increase in NFC and the decrease in hemicellulose/ cellulose and lignin content, since carbohydrates are a source of carbon and energy more available for animals and microorganism than polymers (Dohme et al., 2001). Thus, is reasonable to conclude that the mycelial growth increases the potential to use *Jatropha* seed cake in animal feed.

Therefore, *P. ostreatus* degrades phorbol esters found in *Jatropha* seed cake detoxifying this residue of bio-diesel manufacture and improves the potential to use this residue as ration.

Conflict of Interests

The author(s) have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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Full Length Research Paper

Phytochemical analysis and antimicrobial bioactivity of the Algerian parsley essential oil (*Petroselinum crispum*)

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In this paper, we extracted, analyzed and studied the antimicrobial activity of Algerian parsley essential oil on several microbes that cause infectious diseases and its effects on kinetics of lactic acid production by *Lactobacillus casei subsp. rhamnosus*. The essential oil of parsley (*Petroselinum crispum Hoffm*) obtained by hydrodistillation was characterized by its physicochemical properties and by its chromatographic profiles. Myristicin and dillapiol were identified by gas chromatography-spectrometry mass (GC/MS). The essential oil showed a high antimicrobial spectrum towards *Bacillus cereus* and *Candida albicans*, average effectiveness against *Clostridium perfringens*, *Staphylococcus aureus* and *Enterococcus faecalis* and no influence on *Escherichia coli*. The key odorant effects of parsley in the growth of *Lactobacillus casei subsp. rhamnosus* was studied. The results showed that *L. rhamnosus* can produce up to 10.96 and 13.78 g.L^{-1} of lactic acid on the control fermentation and on the second fermentation, respectively, characterized by the addition of 20 µL of the essential oil in the growth exponential phase.

Key words: Parsley, essential oil, dillapiol, myristicin, antimicrobial activity, *Lactobacillus rhamnosus*.

INTRODUCTION

Essential oil in plants presents great interests in food, pharmaceutical, cosmetic and perfume industries by virtue of their aromatic properties. Parsley (*Petroselinum crispum Hoffm*) belonging to the *Apiaceae* family is considered as an aromatic and medicinal plant used often in traditional medicine for their diuretic, vermifuge, emmenagogue and purgative properties (Lopez et al., 1999; Marczal et al., 1997). Parsley is known for its antidiabetic (Manderfeld et al., 1997), antimicrobial, antihypertensive, anticoagulant, antihyperlipidaemic, anti-hepatotoxic, membrane protective (Fejes et al., 2000) and antioxidant (Nielsen et al., 1999) effects. Phyto-chemical analysis shows the presence of flavonoids, carotenoids, ascorbic acid, myristicin, apiole, terpenoids and coumarins (Anand et al., 1981; Davey et al., 1996; Pino et al., 1997; Tomas et al., 1972; Tunal et al., 1999). The active principles have not yet been fully investigated in food and biotechnology. Researches made were related to the study of stigmasterol, stigmasterol palmi-tate, coumarin, phenol acids, essential oils, carbohy-drates and hydroalcohol extracts (Beaux et al., 1997; Harbone and Saleh, 1971; Hegnauer, 1973; Ravid et al., 1983; Tanira et al., 1996; Trenkle, 1971). The natural extracts stemming from this plant contain a variety of

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phenolic derivatives and essential oils with power inhibittion effect against bacteria.

Lactic acid, a natural organic acid, and its derivatives are widely used in food, pharmaceutical, leather and textile industries (Hujanen and Linko, 1996). Furthermore, since lactic acid has an excellent reactivity derived from both carboxylic and hydroxyl groups, it can undergo a variety of chemical conversions into potentially useful chemicals such as propylene oxide, propylene glycol, acrylic acid, 2,3-pentanedione and lactate ester (Litchfield, 1996; Yun et al., 2003). Recently, there has been an increased interest in L-lactic acid production because it could be used as a raw material for the production of polylactic acid, a polymer used as special medical and environmental-friendly biodegradable plastic, and hence a substitute for synthetic plastics derived from petroleum feedstocks (Amass et al., 1998; Datta et al., 1995).

Antibiotics have been utilized in the farm environment as therapeutic agents and growth promoters for over 50 years (Joerger, 2003). But the emergence of multidrug resistant pathogens and imposed restrictions on the use of antibiotic feed additives have intensified the search for novel possible alternatives (Bedford, 2000; Wierup, 2000; Diep and Nes, 2002; Gillor et al., 2005). In this regard, much interest has been focused on usage of the active principles of plants such as the essential oil, the flavonoids and the alkaloids due to their great potential applications in medicine. In the present work, we investigated for the first time, the determination of physico-chemical properties and identification of major components of the essential oil of parsley stalks and shoots from the region of Mascara (North-west of Algeria). Then, the antimicrobial activity of this essential oil was studied against resistant microbial strains responsible for diseases infections (*Escherichia coli, Bacillus cereus, Staphylococcus aureus, Enterococcus faecalis, Clostridium perfringens* and *Candida albicans*) and the study of the effect of the parsley essential oil addition on the kinetics of the lactic acid production by *Lactobacillus casei subsp. rhamnosus*.

MATERIALS AND METHODS

Essential oil isolation

Fresh aerial part of parsley was collected during April and May 2011 from the region of Mascara, North West of Algeria. The sample (100 g of fresh parsley in 500 mL of water distilled) was submitted to hydrodistillation for 3 h. The distillate was extracted with the methane dichloro and dried over anhydrous sodium sulphate. The resulting essential oil was subsequently analysed.

Identification

The identification of the main constituents of the essential oil of parsley was realized by chromatography (TLC, GC and GC/MS).

Thin layer chromatography (TLC)

On a TLC same patch, we mixed a drop of essential oil of the parsley and a drop of the dillapiol (leading product). Elution was done with ether: petroleum ether (1:4) and visualized using UV, vanillin.

GC and GC-MS analysis

GC-FID

The analysis by GC of the essential oil of parsley was carried out on a Typify VARIAN 3900 gaz chromatograph equipped with a flame ionization detector (FID). A capillary column: [CP-Sil 5CB (30 m x 0.25 mm), DF 0.25 µm], the pulse of the vector gas (N_2), 30 mL/min. The analysis was performed using the following temperature program: 80°C/min at the rate of 20°C/min to 280°C, injector and detector temperature were held, respectively, at 220 and 300°C. The injection volume was 1 µL.

The identification of the dillapiol is made by its co-injection with the essential oil of parsley and with comparison of the retentions indices, back with those of the various peaks revealed in the chromatogram of the studied essential oil.

GC/MS

GC-MS analysis was performed on a gas chromatogram VARIAN SATURN 2100 T interfaced with a mass spectrometer with impact ionization (70eV). A capillary Column [CB 8 (30 M × 0.25 mm), DF 0.25 µm] was used. The column temperature was programmed to rise from 80°C/min to 280°C/min at the rate of 20°C/min. The carrier gas was helium with a flow rate of 1 mL/min, injector and detector temperature were held, respectively, at 280 and 300ºC, the injection volume was 1 µL.

The compound identification was based on their retention indices and mass spectra was compared with the data from the Baser Library.

Antimicrobial activity of the essential oil of parsley

On some microorganisms responsible for diseases infectious

Tested microorganisms: Antimicrobial activity of the essential oil of parsley was tested on 6 clinical strains chosen because of their nosocomial resistance which were *E. coli*, *S. aureus*, *B. cereus*, *C. perfringens*, *E. faecalis* and *C. albicans*. All bacterial strains were provided by the Laboratory of Medical Analysis located in Dr. Yessaâd Khaled Hospital (YKH) of Mascara City, situated in western Algeria. The presumptive identification of these strains was determined by morphological characters and biochemical characterization (Brennan et al., 2001).

The antibiogram: It is the analytical method that allows *in vitro* interpretation of the sensibility of bacteria to antibiotics. Antibiotics selected are: Tetracycline, gentamycine, ofloxacine, ampicillin, penicillin g, clindamycine, oxacilline, chloramphenicol and amoxicillin. We poured a suspension of microorganisms in the limp, dried them at 37°C then deposited the discs of antibiotics in the surface of the agar and incubated at 37°C/24 h. The measure of the diameter of the zones of inhibition allows classifying bacteria in three categories: sensitive, intermediate or resistant (Al-Obeïd, 1991; Joffin and Guey, 1995).

Antimicrobial activity determination: Antimicrobial activity of the parsley essential oil was determined by the agar disc diffusion method. A suspension of each tested micro organism (average concentration is 10^8 cells per mL) was mixed with Mueller Hinton Agar (MHA), then poured on Petri plates with sterilized Whatman No.3 filter paper discs (diameter 6 mm) impregnated with a tested dose of the oil (10, 20, 30 and 40 µL) and were incubated at 37°C for 24 h. The diameters of the inhibition zones were measured (Luu, 2002; Rubio et al., 2003).

Enumeration after culture: The enumeration of the viable bacteria is a collectively used method. Culture is made on agar medium in box. After incubation in a suitable temperature, the number of appeared colonies corresponds to cells present in the volume analysed by the suspension (Meyer and Deiana, 1988).

On the kinetics of the lactic acid production by Lactobacillus casei subsp. rhamnosus

Bacterial strain and media

The *L. casei subsp. rhamnosus* (LBC80 10D) strain used in all our experiments was supplied by the Rhone Poulenc group (Nancy France). It was maintained on MRS medium in the presence of 10% glycerol and stored at -20°C. The MRS medium used in growth and inoculum preparations contained the following components (in $g.L^{-1}$): 10 Soya peptone, 10 beef extract, 5 yeast extract, 2 K_2HPO_4, 5 $NaCH_3CO_2,3H_2O$, 2 triammonium citrate, 0.2 $MgSO_4,7H_2O$, 0.05 $MnSO_4, H_2O$, 15 glucose and 1 mL Tween 80. The pH was adjusted to 6.25 by 25% (w/w) NH_4OH aqueous solution prior to sterilization at 108°C for 15 min. The reactivation phase is realized after two successive transplantations at 42°C during 2 h on liquid MRS medium (Amrane and Prigent, 1994).

Fermentation conditions and methods

All experiments were carried out in a 2 L jar fermenter (Applikon Biocontroller ADI1030) with an initial volume of 1.5 L at 42°C. The agitation speed was set at 200 rpm to insure complete mixing of the fermentation medium. The inocula were incubated at 42°C for 12 h at 200 rpm before their transfer to the fermenter in a 10%. The culture pH was maintained at 6.25 by automatic addition of 25% (w/w) NH_4OH solution using a computer coupled peristaltic pump during the 24 h of fermentation. The samples were withdrawn at desired intervals and frozen for further analysis. The fermentation batch (control) of *L. rhamnosus* for production of L-lactic acid at a glucose concentration of 15 $g.L^{-1}$ was carried out. Other culture led in the same conditions was also prepared containing 20 µL of the parsley essential oil added during the exponential growth phase (this concentration is chosen after preliminary tests). The evolution of the biomass, the residual glucose amount and the lactic acid production are followed in regular time intervals.

Microbiologic method

Gram colouring is made regularly to control at the same time any risk of contagion and the presence of spores.

Analytical methods

The biomass was determined by measurement of the optical density (OD) at 570 nm by a spectrophotometer HITACHI 4-2000. Culture samples were centrifuged (13200 g at 4°C for 5 min), diluted and filtered. Residual glucose and lactic acid concentrations were determined by Multi parameter Medical Analyzer. The enzymatic kit used for the lactic acid dosage is the PAP Ref-61 192 and for the glucose dosage it is the Elitech diagnosis ref - GPSL-0500.

Data processing treatment

The calculation of the fermentation kinetic parameters requires a preliminary data processing (smoothing) of the rough experimental data with the software KALEIDAGRAPH. This data processing is based on the technique of the averages slipping by using a second degree polynomial.

Calculation of the fermentation kinetic parameters

The various analyses carried out allow the following time evolution of the component concentrations present in the culture medium: [Biomass: X(OD) = f (t), sugars: S = f (t) and the metabolite produced (lactic acid): P = f (t)]. From these raw data, it is possible to calculate the fermentation kinetic parameters in the batch culture by the calculation of the volumetric growth rate (r'''_x in $g.L^{-1}.h^{-1}$) and the specific growth rate (µ in h^{-1}):

$$r'''_X = \frac{dX}{dt}, \quad \mu = \frac{r'''_X}{X}$$

The calculation of the volumetric sugar consumption rate ($r'''s$ in $g.L^{-1}.h^{-1}$) and specific sugar consumption rate (Qs in $g.g^{-1}.h^{-1}$) is also possible:

$$r'''_S = -\frac{dS}{dT}, \quad Qs = \frac{r'''_S}{X}$$

as well as the volumetric lactic acid production rate ($r'''p$ in $g.L^{-1}.h^{-1}$) and the specific lactic acid production rate (Ql.a in $g.g^{-1}.h^{-1}$):

$$r'''_P = \frac{dP}{dt}, \quad Ql.a = \frac{r'''_P}{X}$$

The maximal specific growth rate (µmax) was determined from the slopes of the plotted linear curve:

$\ln X/X_0 = f(t)$ (Bimbenet and Loncin, 1995)

Outputs in biomass and lactic acid (the slopes)

The biomass (Yx/s) and products (Yp/s) yields are defined as the mass ratios in biomass and metabolites formed per gram of consumed carbonaceous substrate. During this fermentation, we were interested in the yields of sugar conversion into biomass and lactic acid.

X_0, S_0, L_0, represents the concentrations in respectively biomass, substrate and metabolites (lactic acid) produced at time t_0 of the fermentation and *X, S, L* at time t of the fermentation.

The approach used to determine the outputs consists in plotting linear curves: $[(X–X_0) = f (S_0–S)]$ and $[(L.A–L.A_0) = f (S_0–S)]$. The slopes obtained from these straight lines represent respectively the outputs of sugar conversion into biomass and lactic acid (Djidel, 2007).

RESULTS AND DISCUSSION

Characterization and identification of the parsley essential oil

The essential oil of the parsley was obtained with a yield on 0.14 and 0.08%, respectively, for the dichloromethane and the pentane. This can be explained by the strong solubility of the essential oil in a polar solvent. The isolated essential oil of brown yellow colour and characteristic smell of the parsley was characterized by its physico-chemical indications (Table 1).

Table 1. Physicochemical characterization of parsley essential oil.

Physicochemical parameter	Result
Density	1.17
Rotators power	+2°
Indication of refraction	1.322
Miscibility in the ethanol	18
Indication of iodine	2.03
Indication of acid	11.22
Indication of ester	54.71
Iindication of saponification	46.56

Table 2. The time of retention indices and the percentage of every peak.

Number Pic	Retention indices (min)	Percentage (%)	Products identified
1	6.82	1.6	-
2	7.44	47.5	Dillapiol
3	7.72	12.66	-
4	12.53	38.22	-

Thin layer chromatography (TLC)

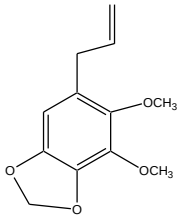
Dillapiol was revealed by TLC by comparison of its factor of keeping back (Rf = 0.57) with those of the essential oil.

Gas chromatography (GC)

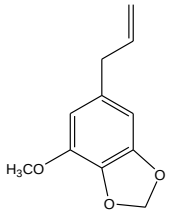
Table 2 gives the time of retention indices and the percentage of every peak. Peaks 1, 3 and 4 were not able to be identified by absence of leading products. The chromatogram (Photo 1), representing the co-injection of the dillapiol with parsley essential oil, indicates the presence of this heterocyclic. Indeed, one observes a net increase of the peak no 2. The percentage passes from 19.11 to 47.5%.

Gas chromatography/spectrometry mass (GC/MS)

With the aim of confirming the presence of the dillapiol, we appealed to the chromatography in phase vapour coupled with the mass (GC/MS). We notice that the spectre of mass of the majority peak is in accordance with the fragmentation of the dillapiol: M +: 222, 207, 191, 177, 149, 121 and 77. Besides, the presence of the myristicin was listed M+: 192, 161, 119, 91 and 65.



Dillapiol



Myristicin

Comparison of the chemical composition of parsley essential oil with other studies

By comparison between our results and those reported in the literature, we are going to quote some examples of the works, which showed the chemical composition of essential oil of parsley. Essential oil of parsley of Germany contains the apiol, the monoterpenes α- and β-pinene, of the myristicin (Spraul et al., 1992).

That of Egypt contains 23.8% of myrcene, 39.7% of myristicin, 6.94% α- pinene, 4.57% β- pinene, 1.11% α-phellandrene, 17.1% 1.3.8 p-menthatriene, 1.03% of dillapiol, 0.71% of bisabolole and 0.11% of camphor (Hashem and Sahab, 1999).

Studies showed the variety of the chemical composition of the essential oil of parsley. The major product is the 1,3,8-p-menthatriene for several foreign oils. Table 3 gives the main constituents of the essential oil of the parsley for several countries (Simon and Quinn, 1988).

Contrary to the foreign oils, the Algerian essential oil of parsley is constituted mainly of dillapiol isomer of the apiol.

Antimicrobial activity of the parsley essential oil

On tested microorganisms

Characterization and identification of the tested microorganisms: On the Mac Conkey medium, *E. coli* suspected colonies presented a diameter from 2 to 3 mm; they are red to pink, smooth, brilliant and flat with regular edges. *E. coli* are Gram negative (Photo 2). The most important biochemical characteristics are presented in Table 4.

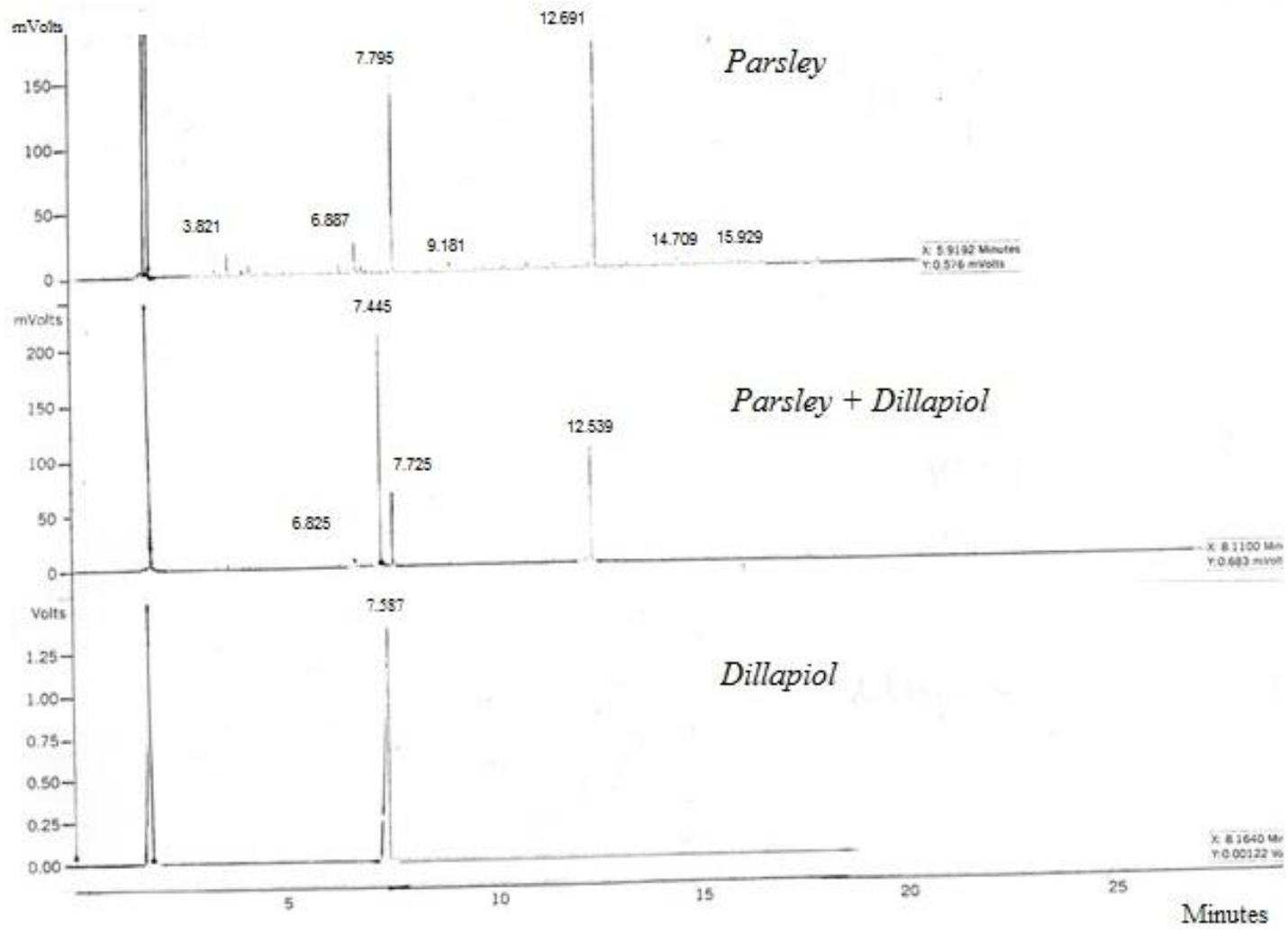


Photo 1. Chromatogram in gaz phase of parsley essential oil.

Table 3. Percentages of the main constituents of the essential oil foreign of Parsley.

Country / Product	Turkey	Arabie Saoudite	Yougoslavie	Iran
α- Pinene	2.3	1.1	0.1	0.6
β- Pinene	1.6	0.8		0.5
Myrcene	9.7	10.4	2.8	2.9
α-Phellandrene	1.1	0.8	0.3	0.9
β-phellandrene	12.1	8.0	6.2	9.7
Terpinene	0.1	0.1		0.2
Terpinolene	2.9	13.9	2.1	5.4
1,3,8-p-Menthatriene	62.8	44.0	20.1	64.7
Thymol		2.0		
Myristicin		1.5	60.5	0.2
Apiol			1.4	

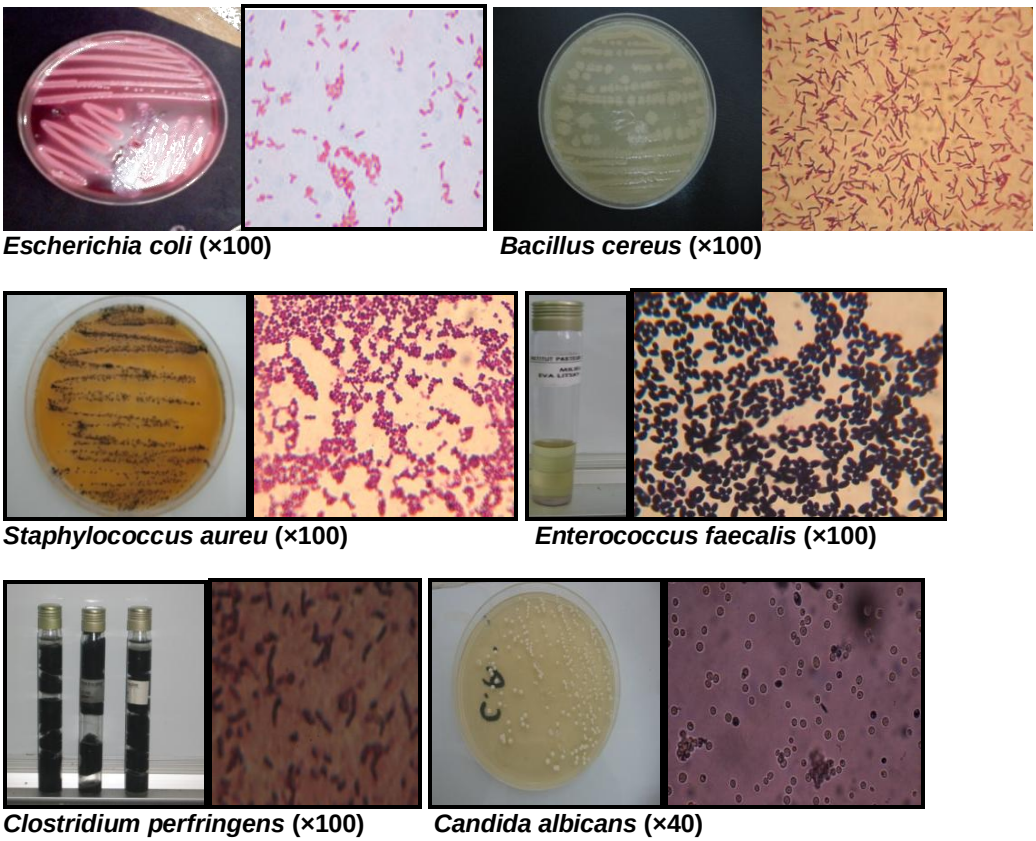


Photo 2. Macroscopic and microscopic aspects of tested microorganisms.

Table 4. Biochemical characteristics of *E. coli.*

Biochemical tests		Results
Oxidase test		-
O.N.P.G.		+
Simmons citrate		-
Reductase nitrate		+
	Lactose	+
	Saccharose	+
	Glucose	+
TSI	Gaz	+
	H_2S	-
	Urea	+
Urea-indol	Indol	+
	Mannitol	+
Mannitol-mobility	Mobility	-
	RM	+
RM/VP	VP	-
ADH/ LDC/ ODC		-/+/+

B. cereus appears under shape of big dry, extensive and irregular colonies of a whitish colour. This microorganism is Gram positive. The most important biochemical characteristics are presented in Table 5.

Characteristic colonies of *S. aureus* are black brilliant 1 to 2 mm in diameter, surrounded with zones of transparency. Microscopic observation shows that these microorganisms are Gram positive. For biochemical tests, we realized for the first time, the tests of catalase and of coagulase which are then supported by the gallery API Staph (Table 6).

In Eva Litsky liquid medium, the faecal Streptococci form a trouble and a purple pastille in the heart of tube. The isolation of *E. faecalis* was made from the faecal Streptococci. Gram colouring shows that these

Table 5. *Bacillus cereus* (characters of determination).

Biochemical test	Result
Catalase	+
Respiratory type	Facultative
Fermentation of sugars (Lactose)	+
Haemolyse	+
Degradation of the casein	+

Table 6. *S. aureus* identification (positive reactions after 24 h at 37°C).

Primordial test				
Catalase			**Coagulase**	
+			**+**	
API Staph.				
0	GLU	FRU	MNE	MAL
-	+	+	+	+
LAC	TRE	MAN	XLT	MEL
+	+	+	-	-
NIT	PAL	VP	RAF	XYL
/	/	/	-	-
SAC	MDG	NAG	ADH	URE
-	-	-	+	+

Table 7. Identification characters of *E. faecalis*.

Biochemical test		Result
Catalase		-
Mannitol-mobility	Mannitol	+
	Mobility	-

Table 8. *C. perfringens* identification.

IND	URE	GLU	MAL	LAC
+	+	+	+	+
SUC	MAN	SAL	XYL	ARA
+	+	+	+	-
GEL	ESC	GLY	CEL	MNE
+	+	-	+	+
MLZ	RAF	SOR	RHA	TRE
-	+	+	+	+

microorganisms are pods and Gram positive (Photo 2). Two biochemical tests were realized: the catalase and mannitol-mobility (Table 7).

The colony suspect of *C. perfringens* is a black colony of a diameter superior to 0.5 mm. A microscopic morphology reveals that these microorganisms are bacilli to positive Gram. Adding to the test of the catalase, *C. perfringens* was identified by using a miniaturized technique called the gallery API 20 A (Table 8).

C. albicans cultured on the selective medium Sabouraud gives white, creamy and smooth colonies. The simple colouring of blue in the lactophenol shows the presence of the egg-shaped cells (Photo 2).

The antibiotic sensitivity testing: The results of the antibiogram (Table 9 and Photo 3), showed that the *E. coli* strain was sensitive to gentamycine and to ofloxacine, it can be treated in a usual and intermediate dose in tetracycline and ampicillin. According to Bachir and Benali (2008), this strain is sensitive to chloramphenicol, resistant to ampicillin, doxycycline and pristinamycine and intermediate resistant to erythromycin and nitroxoline. According to the results obtained, we can classify *B. cereus* in two categories of sensitivity (intermediate and sensitive). *B. cereus* is sensitive to gentamycine, tetracycline and ofloxacine. It is treated in a usual and intermediate dose in penicillin G and clindamycine. It is can be treated by the increase of the dose of the penicillin G or clindamycine. *S. aureus* is intermediate in the penicillin G and sensitive to gentamycine, clindamycine, tetracycline and oxacilline. *E. faecalis* is resistant to all the chosen antibiotics. *C. perfringens* is sensitive to chloramphenicol and tetracycline. It is called intermediate in the penicillin G in amoxicillin and in ampicillin.

Antimicrobial activity of the parsley essential oil: According to the results of aromatogram obtained in Table 10 and Photo 4, we can note that the various doses applied to the *B. cereus, S. aureus* and *C. perfringens* presents an inhibitory activity increasing to 10, 20 and 40 µL. The doses of 10 and 20 µL have an identical effect on the *E. faecalis* representing a diameter of 14 mm. *C. albicans* countered sensitive and *E. coli*, showed insensitivity against the action of the various doses of the *Petroselinum crispum* essential oil.

We noticed that the *E. coli*, which is a bacterium to negative Gram, showed insensitivity to the action of the various doses of the *P. crispum* essential oil, contrary to bacteria that was Gram positive. This result is supported by the variation of the result by the different composition and the cellular structure of bacteria to Gram positive and negative bacteria, because and according to Larpent and Larpent-Gourgaud (1985), the Gram negative bacteria cells contain a wall rich in lipids and a double membrane equipped with a died plasmic space. This confers her degree of protection against the constituents of the essential oil. So, we supposed that *E. coli* inhibition requires high concentrations of essential oil. A lessening spectre of activity was observed with the studied essential oil which represents a good activity on the bacterial strain *B. cereus* and *C. albicans* with a diameter of zone of inhibition, respectively of 26 and 24 mm in means and a weak activity on *S. aureus* and *C. perfringens* which represent a diameter of zone of inhibition of 18.5 mm in mean and finally the *E. faecalis* with an average of zone of inhibition of 17 mm. This sensibility can be due to the action of one or several essential oil of parsley.

The lactic acid production by L. casei subsp. rhamnosus

The results of the two batch fermentations of *L. rhamnosus* control and with 20 µL of parsley essential oil are presented in Figures 1 to 3, respectively. We noticed the increase in the concentrations of biomass, residual

Table 9. Antibiogram results of tested microorganisms with traditional antibiotics.

Antibiotic	*E. coli*		*B. cereus*		*S. aureus*		*E. faecalis*		*C. perfringens*	
	IZD	R	IZD	R	IZD	R	IZD	R	IZD	R
Tetracycline	8	I	30	S	32	S	0	R	22	S
Gentamycine	20	S	20	S	18	S	-	-	-	-
Ofloxacine	20	S	27	S	-	-	-	-	-	-
Ampicillin	7	I	-	-	-	-	0	R	8	I
Penicillin G	-	-	9	I	10	I	0	R	8	I
Clindamycine	-	-	9	I	20	S	0	R	-	-
Oxacilline	-	-	-	-	16	S	-	-	-	-
Chloramphenicol	-	-	-	-	-	-	0	R	31	S
Amoxicillin	-	-	-	-	-	-	-	-	13	I

IZD: Inhibition zone diameter (mm); R: results.

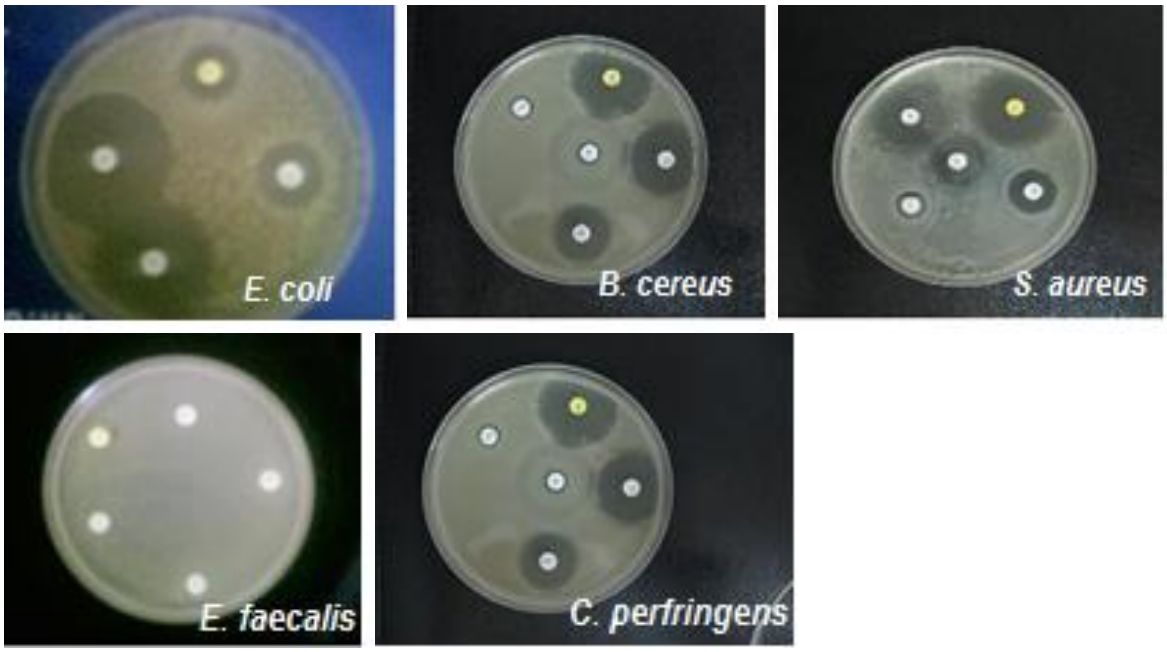


Photo 3. Antibiogram of tested microorganisms.

Table 10. Zone of inhibition (mm) of tested microorganisms in the presence of various doses of the parsley essential oil.

Doses of parsley EO / Inhibition zone diameter (mm)	10 µL	20 µL	30 µL	40 µL
Escherichia coli.	0	0	0	0
Bacillus cereus	23	27	-	29
Staphylococcus aureus	13	20	22	24
Enterococcus faecalis	14	14	16	20
Clostridium perfringens	12	18	21	25
Candida albicans	18	20	24	30

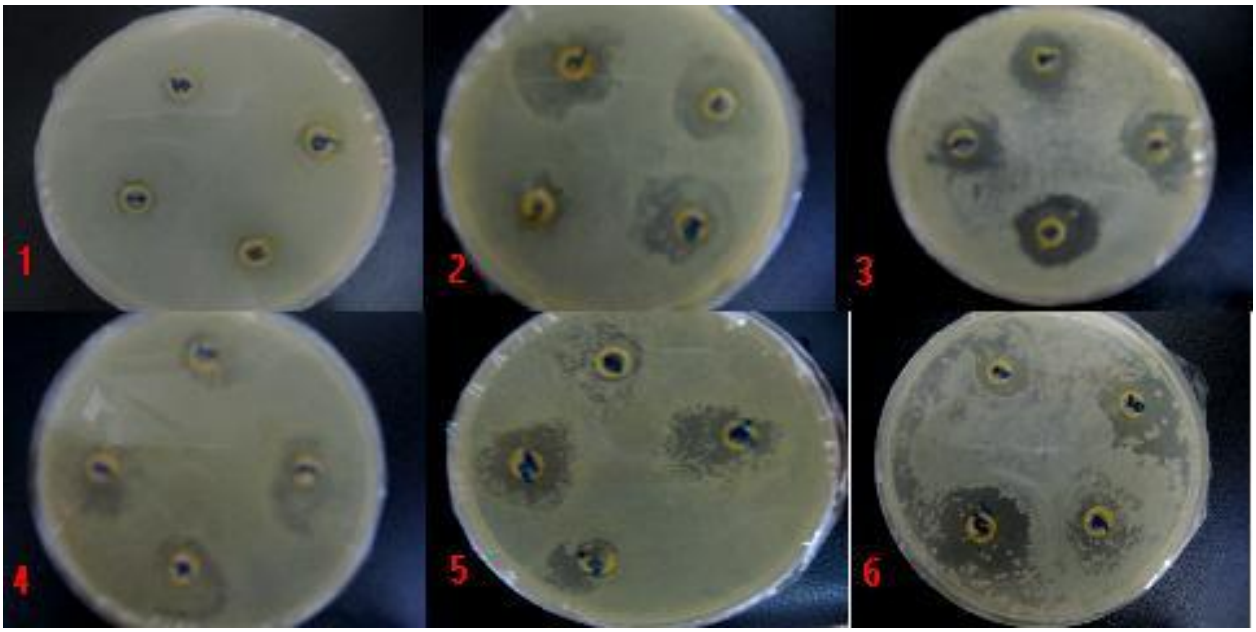


Photo 4. Aromatograms made on microorganisms tested in the presence of various doses of the essential oil of parsley. 1. *Escherichia coli*, 2. *Bacillus cereus*, 3. *Staphylococcus aureus*, 4. *Enterococcus faecalis*, 5. *Clostridium perfringens*, 6. *Candida albicans*.

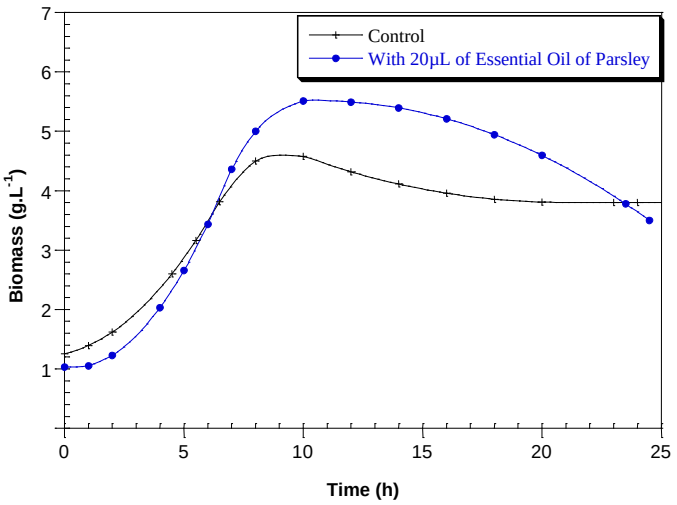


Figure 1. Biomass production during the culture of *Lactobacillus rhamnosus* in two batch fermentations (control with 20 µL of essential oil of parsley).

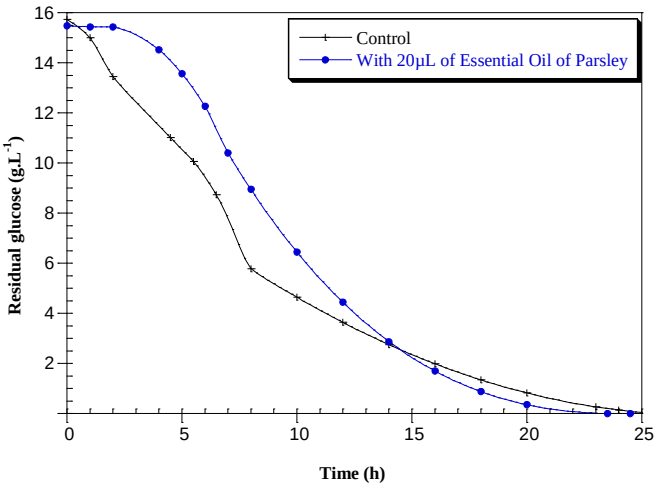


Figure 2. Residual glucose during the culture of *L. rhamnosus* in two batch fermentations (control with 20 µL of essential oil of parsley).

sugars and lactic acid. The initial concentration in glucose (15 $g.L^{-1}$) was the same for the samples. They were inoculated with the same quantity of biomass (1.2 $g.L^{-1}$). According to the obtained results, it was observed that the *L. rhamnosus* growth for the batch fermentations is characterized by a short duration of the latency phase which indicates that the inoculated cells were in full exponential phase.

For the control fermentation, a weak biomass initial concentration of 1.2 $g.L^{-1}$ was observed which increases after 6 h of fermentation until 3.5 $g.L^{-1}$, then until 4.575 $g.L^{-1}$ after 8 h of culture (at the end of the exponential phase). The growth end is due to the glucose exhaustion in the culture medium which reaches a final value of 0.008 $g.L^{-1}$ after 24 h of fermentation. During the stationary phase (after 8 h of culture), the strain consumed the glucose and used it only for the cellular maintenance. In parallel, the lactic acid production started from a value

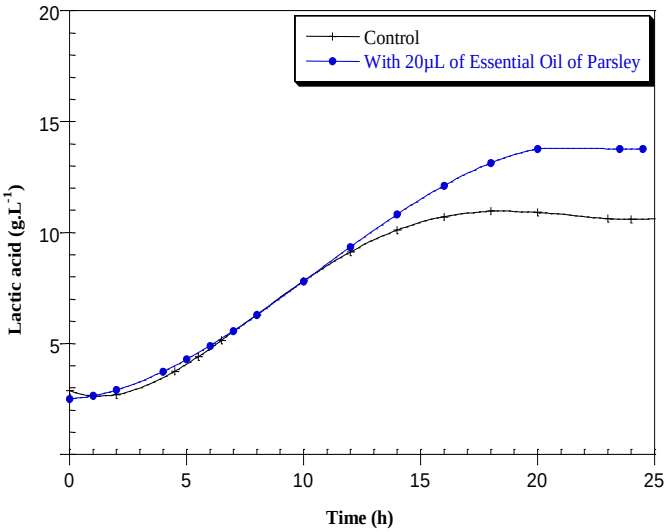


Figure 3. Lactic acid production by *L. rhamnosus* during two batch fermentations (Control with 20 µL of essential oil of parsley).

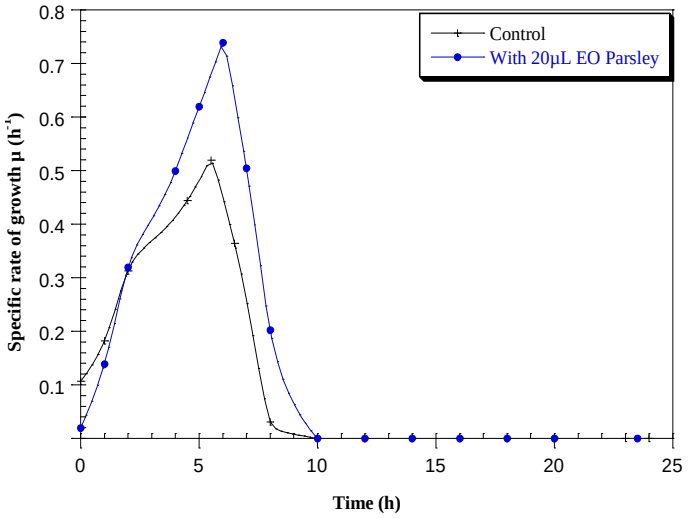


Figure 4. Specific rate of growth of *L. rhamnosus* during two batch fermentations (control with 20 µL of essential oil of parsley).

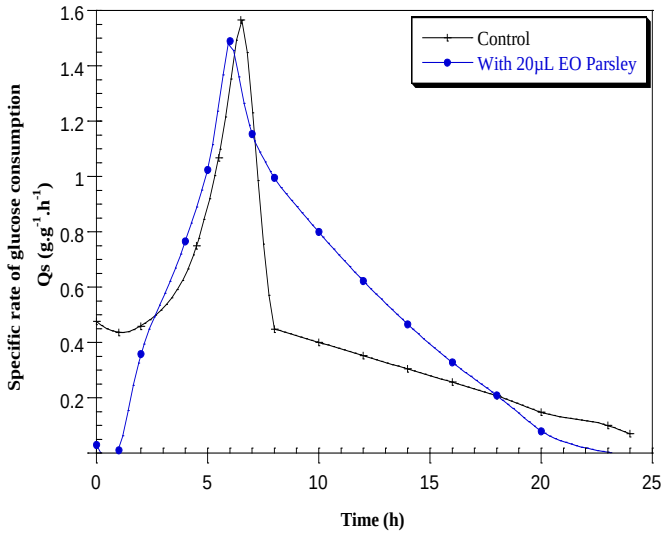


Figure 5. Specific rate of glucose consumption by *L. rhamnosus* during two batch fermentations (control with 20 µL of essential oil of parsley).

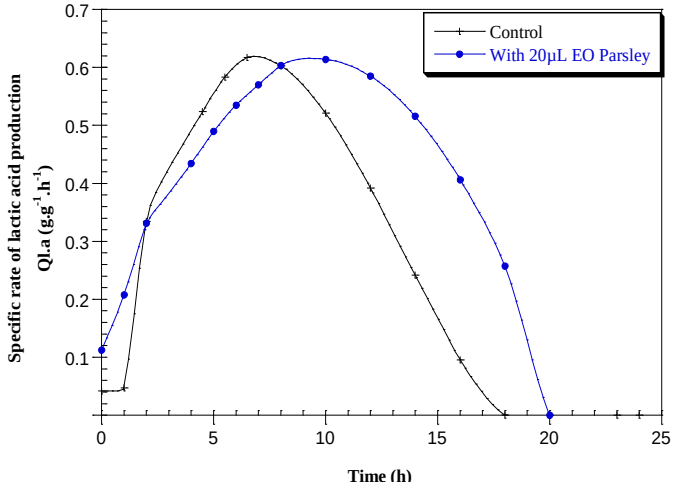


Figure 6. Specific rate of lactic acid production by *L. rhamnosus* during two batch fermentations (control with 20 µL of essential oil of parsley).

of 3 g.L^{-1} and reached 10.96 g.L^{-1} of lactate after 24 h of fermentation. During this fermentation that lasted for 24 h, the quantity of consumed sugar was 15 g.L^{-1}.

However, for the batch fermentation, the *Lb. rhamnosus* biomass started with an initial concentration of 1.2 g.L^{-1} and the parsley essential oil was added during the full exponential phase after 6 h of culture corresponding to a biomass quantity of 3.5 g.L^{-1}. After 10 h of fermentation, cellular concentration increased at a value of 5.51 g.L^{-1}. For the production of lactic acid, the *L. rhamnosus* culture reached the maximum value of 13.78 g.L^{-1} after 24 h of fermentation.

The specific rate of *L. rhamnosus* growth (µ) in the control MRS medium started at 0.11 h^{-1} and reached its maximum value of 0.5 h^{-1} after 6 h of fermentation, then it decreased to 0 h^{-1} after 10 h of culture. For the parsley fermentation, µ started with an initial value of 0.02 h^{-1} increasing to 0.74 h^{-1} after 6 h of fermentation (Figure 4).

The maximal sugar consumption specific rate (Qs max) was 1.5 g.g^{-1}.h^{-1} for the control and parsley and the maximal (Ql.a.max) lactic acid production specific rate was of 0.61 g.g^{-1}.h^{-1} for the control as well as parsley cultures (Figures 5 and 6).

The growth kinetics may be characterized by a maximal growth specific rate (µmax) which is equal to 0.17 h^{-1} in the MRS medium (control) and increases to 0.22 h^{-1} in the

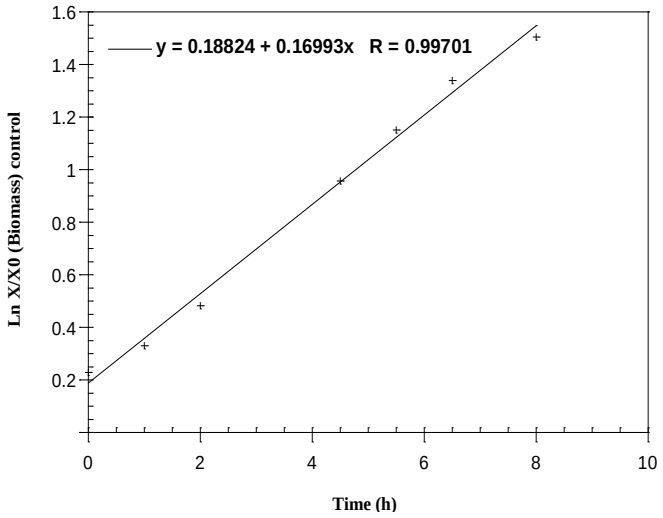


Figure 7. Maximal specific rate of growth µmax (slopes) during batch fermentation (control).

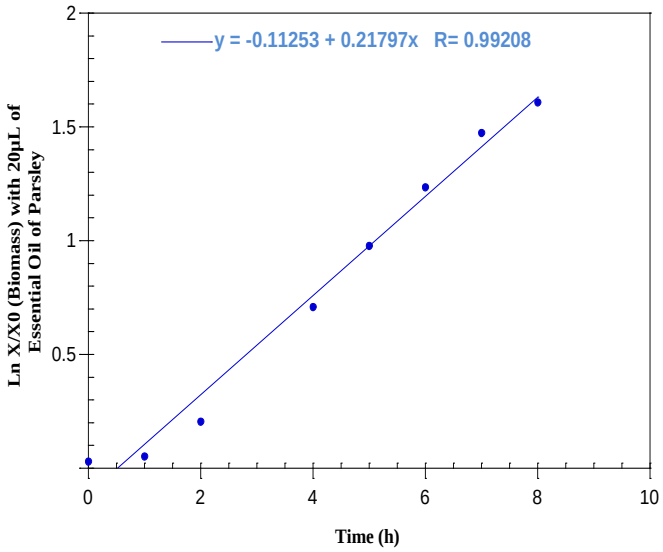


Figure 8. Maximal specific rate of growth µmax (slopes) during batch fermentation (control with 20 µL of essential oil of parsley).

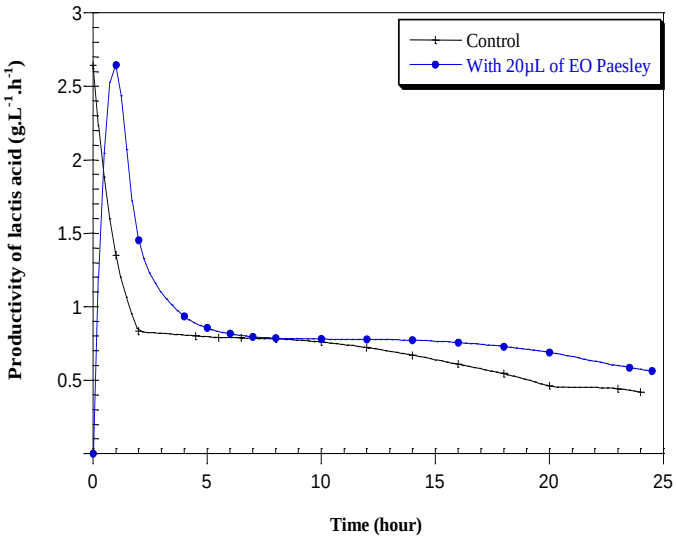


Figure 9. Productivity of lactic acid in two batch fermentations (control with 20 µL of essential oil of parsley).

the presence of the 20 µL of parsley essential oil. The lactic acid productivities are identical for both control and parsley cultures (Figures 7 to 9 and Table 11).

For the control fermentation, the yield of sugar conversion to biomass was 0.84 [1] and 0.71 $g.g^{-1}$ into lactic acid. It seems that the majority of the sugar is used for the cellular maintenance and multiplication and the remainder for the lactic acid production. The addition of the parsley essential oil in the culture stimulates the lactic acid production with a conversion yield of 0.87 $g.g^{-1}$.

According to the results in Table 11, one finds a positive correlation between the lactic acid production and the sugar consumption rate. This result shows the fate of consumed sugar: the majority of the consumed sugar is used for the lactic acid production (Table 11).

Conclusion

Medicinal plants represent a plentiful source of bioactive natural substances among which are the essential oils. This work aims to characterize and estimate antimicrobial activity of these substances on *E. coli, B. cereus, S. aureus, E. faecalis, C. perfringens and C. albicans*. The dillapiol constitutes 47.5% of the essential oil of parsley. The identification could be made by the use of chromatographic methods. Besides, the existence of the myristicin in essential oil of parsley was confirmed. It is shown that ethers-oxides are bi-functional having a function of dioxymethylene and one or two functions as methoxylic, found in our essential oil, and possess numerous therapeutic actions. They show antalgic and antispasmodic properties and anti-infectious power comparable to phenols methyl-ethers. *In vitro* analyses, allowed us to determine the antimicrobial effect of essential oil of *P. crispum* by the agar disc diffusion method which turned out more active especially on *B. cereus* and *C. albicans* (*C. perfringens*, *S. aureus* and *E. faecalis* are less sensitive), while it did not have any effect on the *E. coli*. The results of estimation of the effect of the parsley essential oil over the kinetics of lactic acid production by *L. rhamnosus* show a stimulating effect of the *P. crispum Hoffm* essential oil on the kinetics of lactic acid production and growth by *L. rhamnosus*.

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We gratefully acknowledge Pr. J.M. Pons from reactivity

Table 11. Kinetics parameters of batch fermentations.

Fermentation / Parameter	Control	With 20µL of essential oil of parsley
Biomass max ($g.L^{-1}$)	4.57	5.51
Lactic acid max ($g.L^{-1}$)	10.96	13.78
Residual glucose ($g.L^{-1}$)	0.008	0.007
µmax (h^{-1})	0.17	0.22
Qsmax ($g.g^{-1}.h^{-1}$)	1.56	1.50
Q.l.a max ($g.g^{-1}.h^{-1}$)	0.61	0.61
Yx/s ($g.g^{-1}$)	0.84	0.41
Yp/s ($g.g^{-1}$)	0.71	0.87
Productivity in lactic acid ($g.L^{-1}.h^{-1}$)	2.64	2.64

µmax: Maximal specific rate of growth; Qs.max: maximal specific rate of consumption of sugars; Ql.a. max: maximal specific rate of production of lactic acid; Yx/s: yield on conversion of sugars in biomass; Yp/s: yield on conversion of sugars in lactic acid.

in organic synthesis of Paul Cezanne University Aix-Marseille and Pr. M. Fick (ENSAIA, INPL, France).

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African Journal of Microbiology Research

Full Length Research paper

Assessment of detoxification of malathion by *Pseudomonas* isolates

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Malathion degrading bacteria were isolated from a soil sample. Growth of mono and co-cultured strains of *Pseudomonas* in media containing the organophosphate insecticide, malathion as sole source of carbon was employed to assess degradability of the bacteria. On the seventh day of inoculation, bacteria free culture fluids as well as un-inoculated fluids were analyzed by gas chromatography and mass spectrometry (GC-MS). Malathion was not detected in the cell free culture fluids, instead four degradation products namely phoratoxon sulfone, malaoxon, butanedioic acid and ethyl methyl-methyl phosphonate made their appearance in the cultivation culture. All of the three strains used in this study, appeared efficient in degrading the insecticide especially in co-culturing conditions. These bacteria are promising for detoxifying the insecticide contaminated soils.

Key words: *Pseudomonas*, detoxification, malathion.

INTRODUCTION

Malathion [diethyl 2-{(dimethoxyphosphorothioyl) sulfanyl} butanedioate] is a ten carbon compound used widely in agriculture and in public health pest control programs (US EPA, 2013), while in US, it is the most com-monly used organophosphate insecticide (Bonner et al., 2007).

Following application, malathion may be detected on plant surfaces up to 9 weeks (Delmore and Applelhans, 1991), yet with low persistence (Extoxnet, 2000) and rapid degradation in soil (Howard, 1991; Brenner, 1992). It is readily metabolized into even more toxic malaoxon in human body following absorption or injestion. Long-term oral exposure of malaoxon in rats has been found to be 61 times more toxic than malathion (Edwards, 2006). Despite lack of reliable information on adverse health effects of malathion (ATSDR, 2008), it has been found to induce oxidative stress in erythrocytes and alteration of the cellular antioxidant defense system (Durak et al., 2009). However, it is cleared from the body in three to five days (Maugh, 2010).

Bacteria capable of malathion degradation have been well documented by various workers (Shan et al., 2009; Hamouda et al., 2013). This paper reports degradability of *Pseudomonas* isolates from chronically insecticide influenced soil.

MATERIALS AND METHODS

Three strains of *Pseudomonas* were isolated from soil in the vicinity of an insecticide formulating unit on a selective medium. This medium contained commercial grade malathion (50%) as sole source of carbon as well as K_2HPO_4 (dipotassium hydrogen phosphate), $MgSO_4$ (magnesium sulfate), NH_4NO_3 (ammonium nitrate), $FeSO_4.7H_2O$ (hydrated ferrous sulfate), $CaCl_2$ (calcium chloride), copper nitrate, $MnCl_2$ (manganese chloride),

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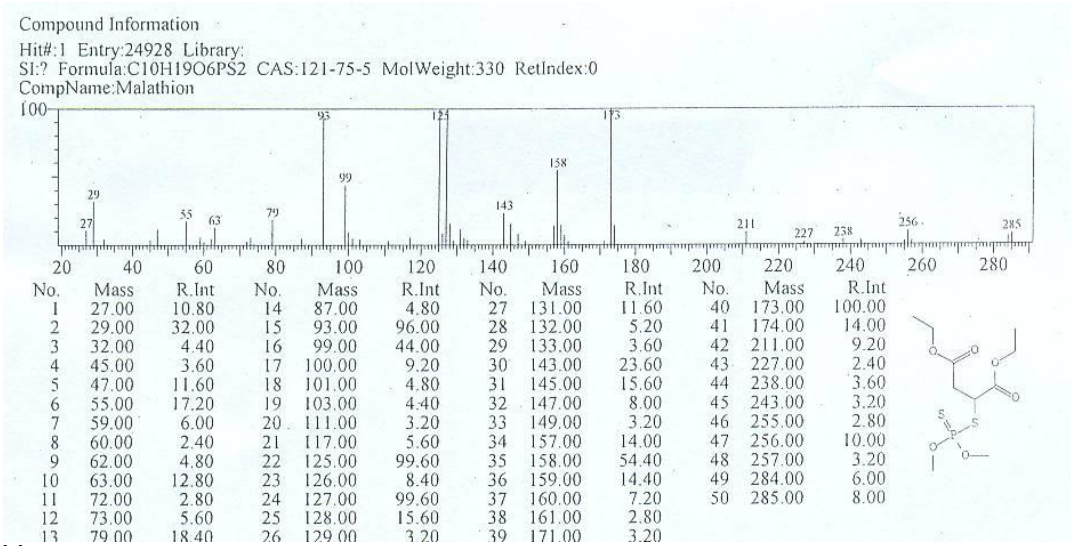
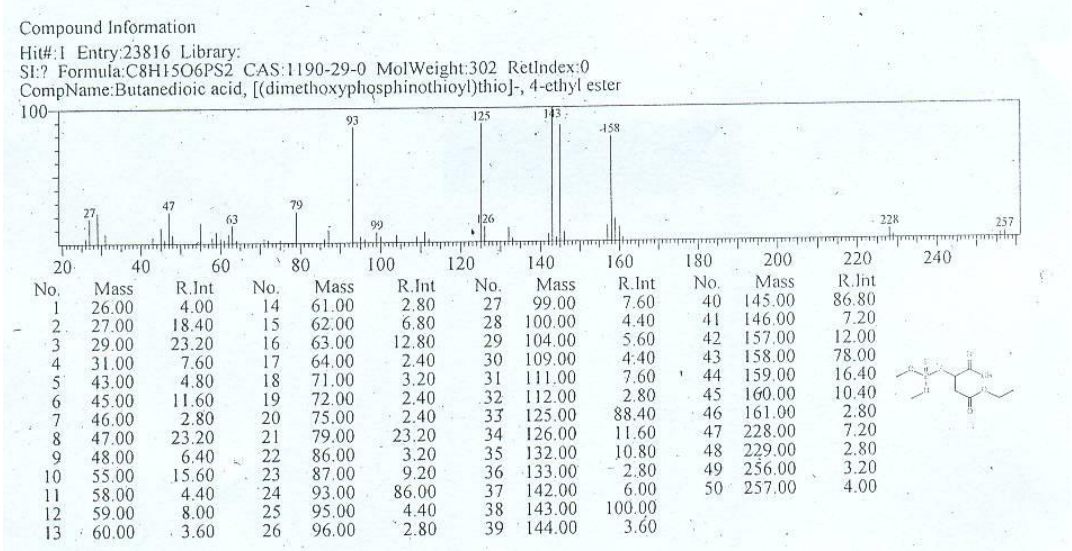


(a)



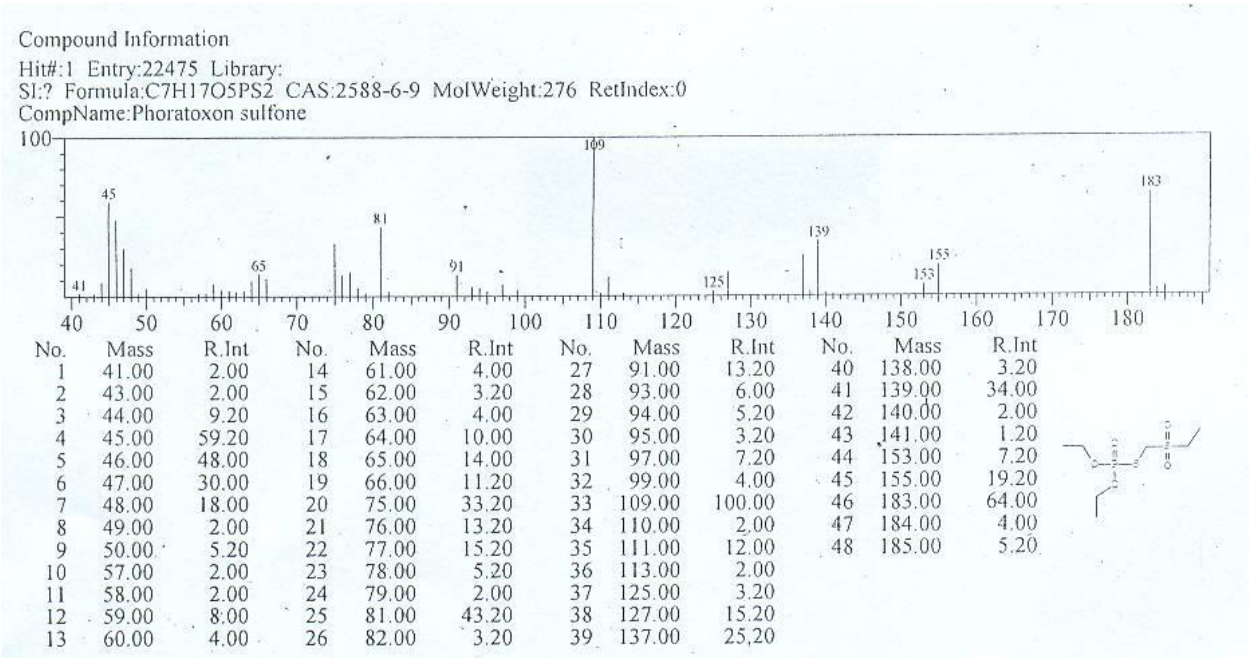
(b)

Figure 1. Chromatogram showing the presence of some molecules (a) Malathion (b) Butanedioic acid (c) Phoratoxon Sulfone and (d) Ethylmethyl methyl phosphonate.

zinc powder and agar (Andleeb et al., 2013). This medium was inoculated by mono and co-cultures of bacterial isolates at their optimum growth conditions with 5% inoculums size for *Pseudomonas aeruginosa* strain MY 06 and 10% inocula size for *P. aeruginosa* and *P. aeruginosa* strain SWD. Cultured fluids as well as un-inoculated control were sampled on seventh day of inoculation filtered through 0.2 µm Millipore filters (Sartorius). The filters were analyzed by using technique GC-MS, available in HEJ (Hussain Ebrahim Jamal), Research Institute of Chemistry, University of Karachi. The column DB-5(optima-5) 30 m x 0.25 mm was used with injection volume of 1.00 µl and flow rate of 1.00 ml/min. Start temperature was 60°C for 5 min and end temperature was 280°C for 1 min. Retention time was fixed at 15-35 min for detection of different compounds. An Agilent 6890 NGC was interfaced with a VG Analytical 79-250s double focusing mass spectrometer. MS operating conditions were ionization voltage 70 eV and ion source 250°C. GC was fitted with a 30 m x 0.32 mm fused capillary silica column coated with DB-5. Data were acquired by means of GC solution software (Shimadzu). Detection and identification of the insecticide and its metabolites produced as a result of bacterial metabolism was accomplished by consulting and comparing data with Standard Molecule library available at Department of Chemistry, Government College University, Lahore (Figure 1).

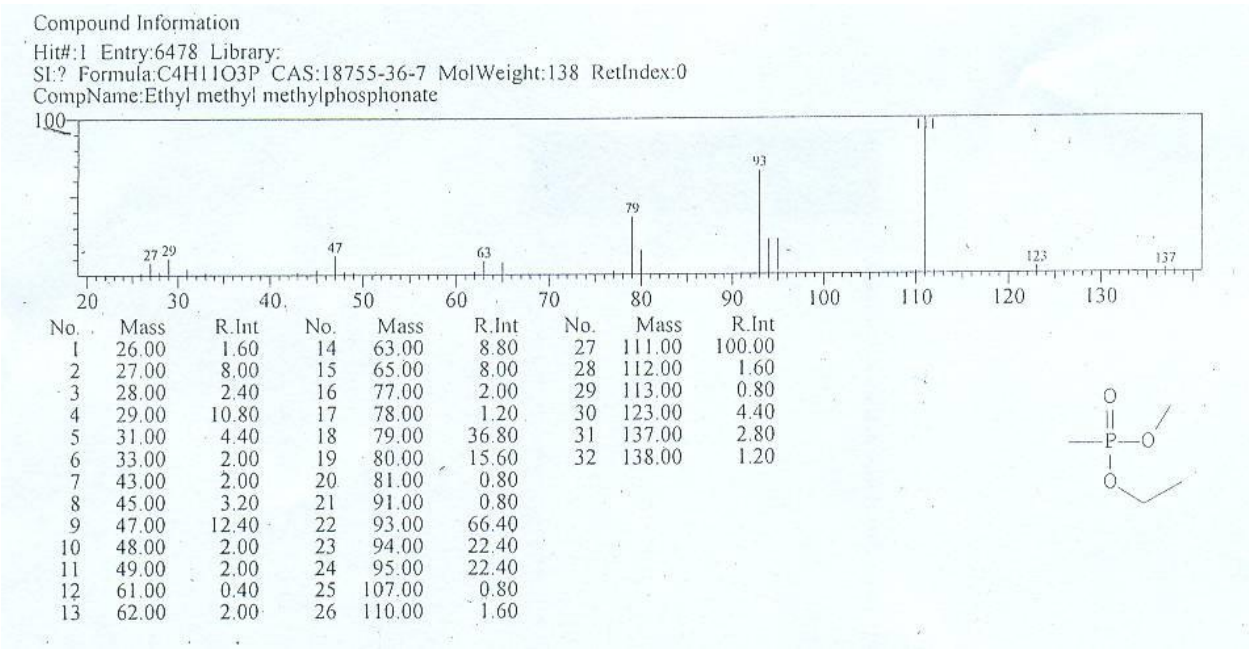
RESULTS

The GC/MS analysis detected the presence of malathion



No.	Mass	R.Int	No.	Mass	R.Int	No.	Mass	R.Int	No.	Mass	R.Int
1	41.00	2.00	14	61.00	4.00	27	91.00	13.20	40	138.00	3.20
2	43.00	2.00	15	62.00	3.20	28	93.00	6.00	41	139.00	34.00
3	44.00	9.20	16	63.00	4.00	29	94.00	5.20	42	140.00	2.00
4	45.00	59.20	17	64.00	10.00	30	95.00	3.20	43	141.00	1.20
5	46.00	48.00	18	65.00	14.00	31	97.00	7.20	44	153.00	7.20
6	47.00	30.00	19	66.00	11.20	32	99.00	4.00	45	155.00	19.20
7	48.00	18.00	20	75.00	33.20	33	109.00	100.00	46	183.00	64.00
8	49.00	2.00	21	76.00	13.20	34	110.00	2.00	47	184.00	4.00
9	50.00	5.20	22	77.00	15.20	35	111.00	12.00	48	185.00	5.20
10	57.00	2.00	23	78.00	5.20	36	113.00	2.00			
11	58.00	2.00	24	79.00	2.00	37	125.00	3.20			
12	59.00	8.00	25	81.00	43.20	38	127.00	15.20			
13	60.00	4.00	26	82.00	3.20	39	137.00	25.20			

(c)



No.	Mass	R.Int	No.	Mass	R.Int	No.	Mass	R.Int
1	26.00	1.60	14	63.00	8.80	27	111.00	100.00
2	27.00	8.00	15	65.00	8.00	28	112.00	1.60
3	28.00	2.40	16	77.00	2.00	29	113.00	0.80
4	29.00	10.80	17	78.00	1.20	30	123.00	4.40
5	31.00	4.40	18	79.00	36.80	31	137.00	2.80
6	33.00	2.00	19	80.00	15.60	32	138.00	1.20
7	43.00	2.00	20	81.00	0.80			
8	45.00	3.20	21	91.00	0.80			
9	47.00	12.40	22	93.00	66.40			
10	48.00	2.00	23	94.00	22.40			
11	49.00	2.00	24	95.00	22.40			
12	61.00	0.40	25	107.00	0.80			
13	62.00	2.00	26	110.00	1.60			

(d)

Figure 1. Contd.

in untreated samples (Figures 2 and 3), whereas malaoxon, butanedioic acid, phoratoxon sulfone and ethyl-methyl phosphonate were detectable metabolites in the fluids obtained after bacterial growth (Table 1; Figures 4 to 6).

As can be seen from the Table 1 the isolate *P. aeruginosa* yielded the four metabolites while the isolates designated as *P. aeruginosa* MY06 detoxified malathion into ethyl methyl methyl phosphonate. The isolate *P. aeruginosa* SWD also yielded only one metabolite, that

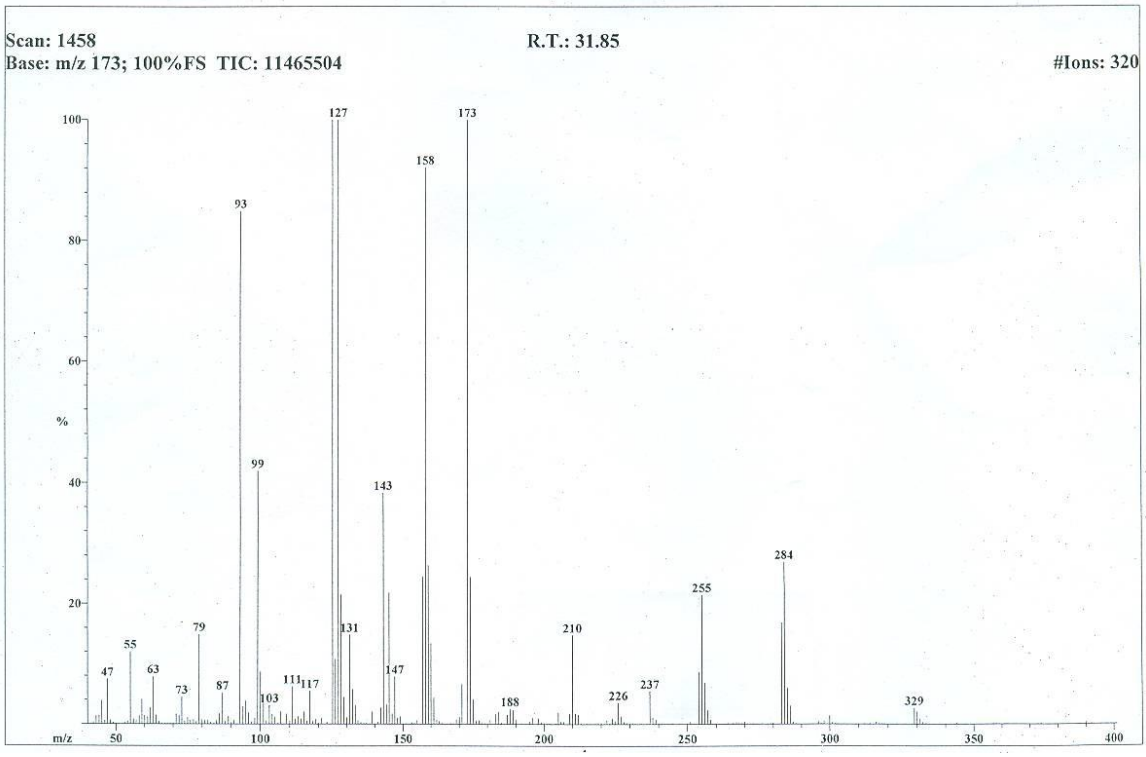


Figure 2. Chromatogram (GC-MS) showing detection of malathion in technical grade used in the study.

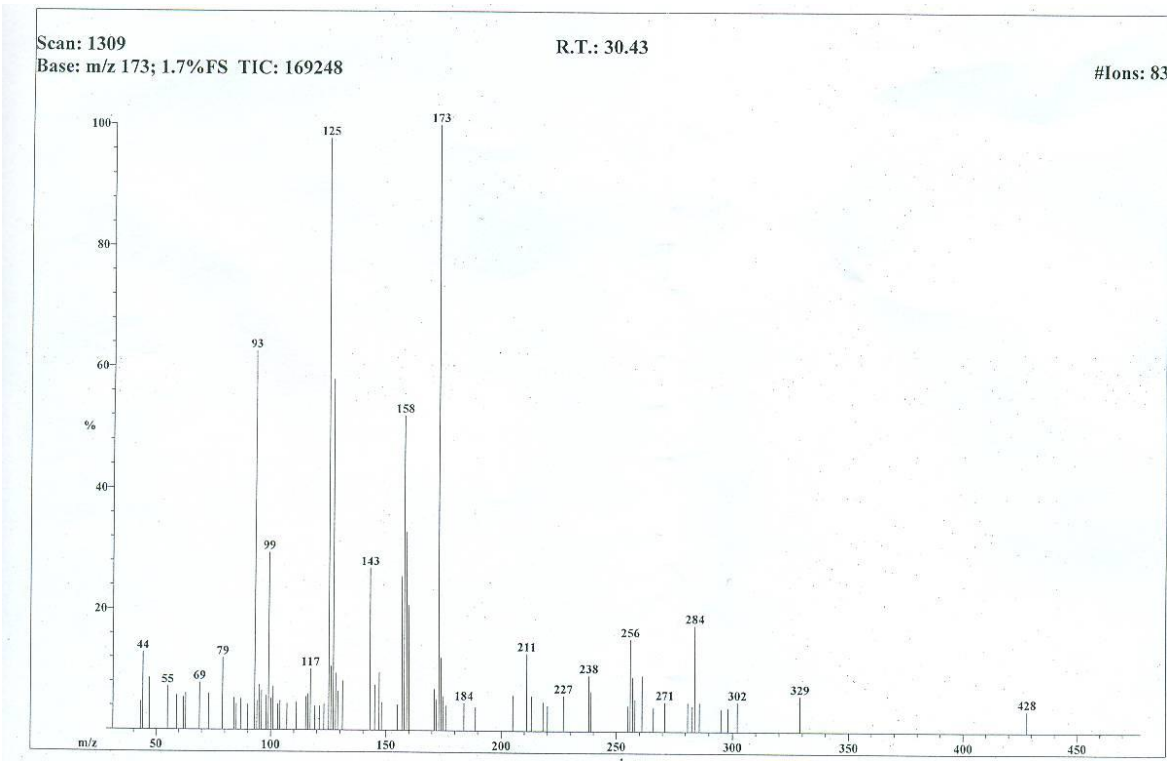


Figure 3. Chromatogram showing GC-MS of 0.5% malathion un-inoculated (control).

Table 1. Metabolites produced by bacterial isolates while growing for 7 days in selective medium containing 0.5% malathion.

Bacterial Isolate	Metabolites			
	Malaoxon (Mol. wt. 314)	Butanedioic acid (Mol. wt. 302)	Phoratoxon sulfone (Mol. wt. 276)	Ethyl methyl methyl-phosphonate (Mol. wt.138)
P. aeruginosa	+ve	+ve	+ve	+ve
P. aeruginosa MY06	-ve	-ve	-ve	+ve
P. aeruginosa SWD	-ve	+ve	-ve	-ve
Poly-culture of above three isolates	+ve	-ve	-ve	+ve

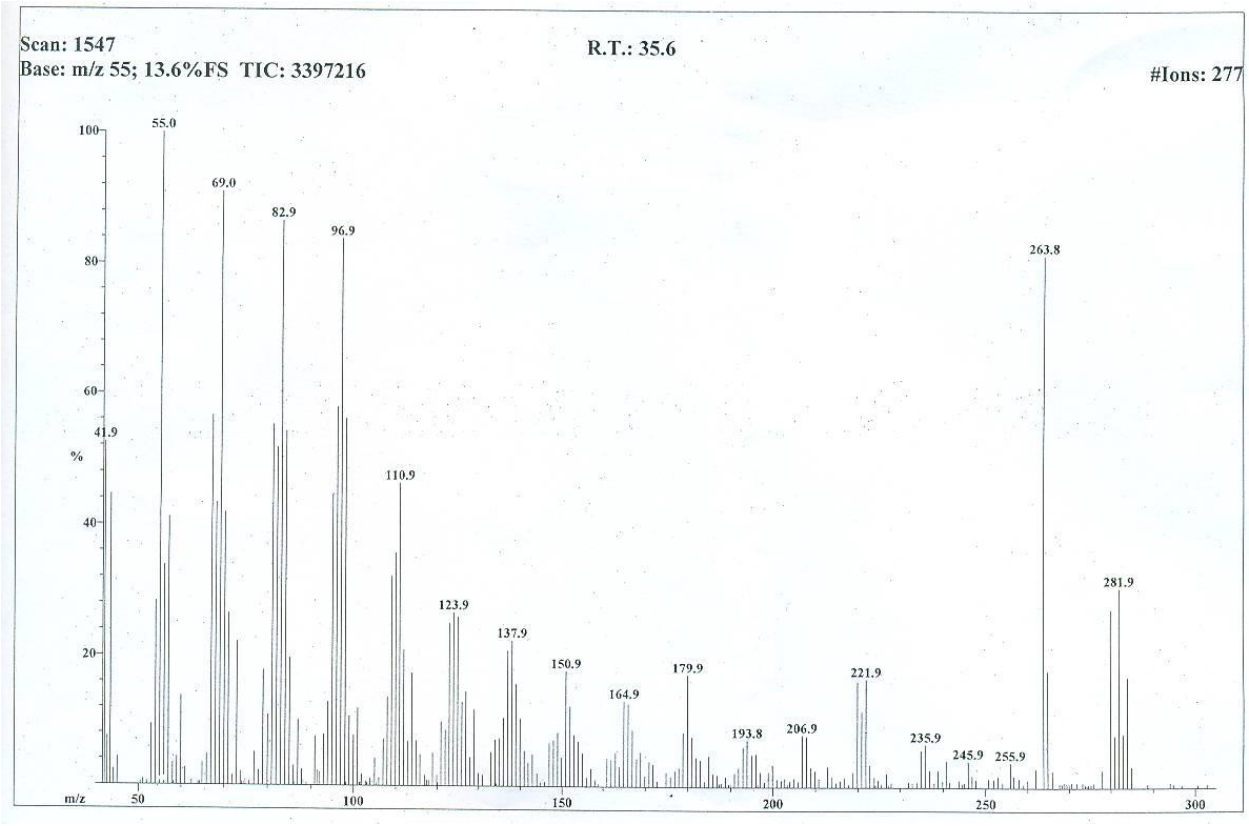


Figure 4. Chromatogram (GC-MS) presenting analysis of a cell-free sample of malathion (concentration 0.5%) inoculated with *P. aeruginosa* strain MY06, indicating absence of malathion and presence of ethyl methyl methyl phosphonate.

is, butanedioic acid. Under co-culture conditions (Figure 6), only malaoxon and ethyl methyl methyl phosphonate were produced.

DISCUSSION

In this study, degradation of malathion has been observed by three isolates of *Pseudomonas*. Various degradation products of malathion, malaoxon, butanedioic acid, phoratoxon sulfone and ethylmethylmethyl phosphonate were detectable. The differential ability of the isolates to yield different sets of the four metabolites reflects their genomic diversity that appeared either in a metabolic scarcity or complete consumption of the non-detectable product by the bacterial isolate. Very interesting observation came from the results of co-culturing experiments, that is, butanedioic acid and phoratoxon sulfone were non-detectable by the analytical techniques employed. This result suggests the change in gene activity of *P. aeruginosa* and *P. aeruginosa* SWD involved in detoxification of malathion. This might had been possible due to

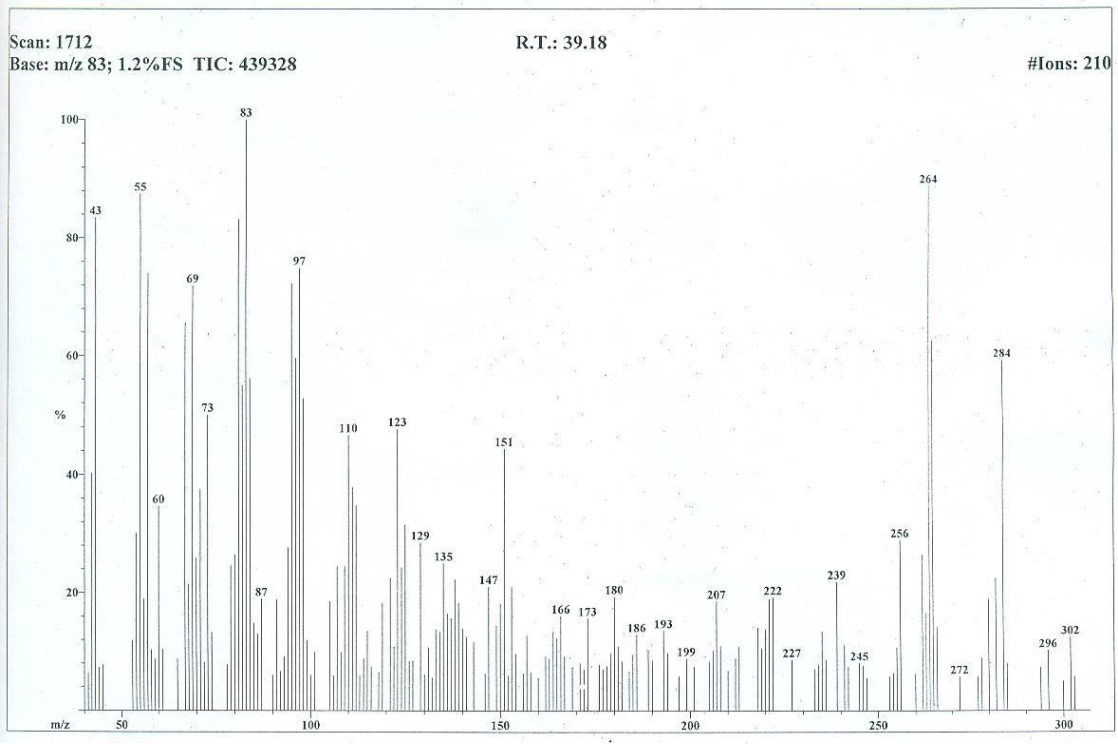


Figure 5. Chromatogram (GC-MS) presenting analysis of cell free sample of malathion (0.5%) inoculated with *P. aeruginosa* strain SWD indicating absence of malathion and presence of butanedioic acid.

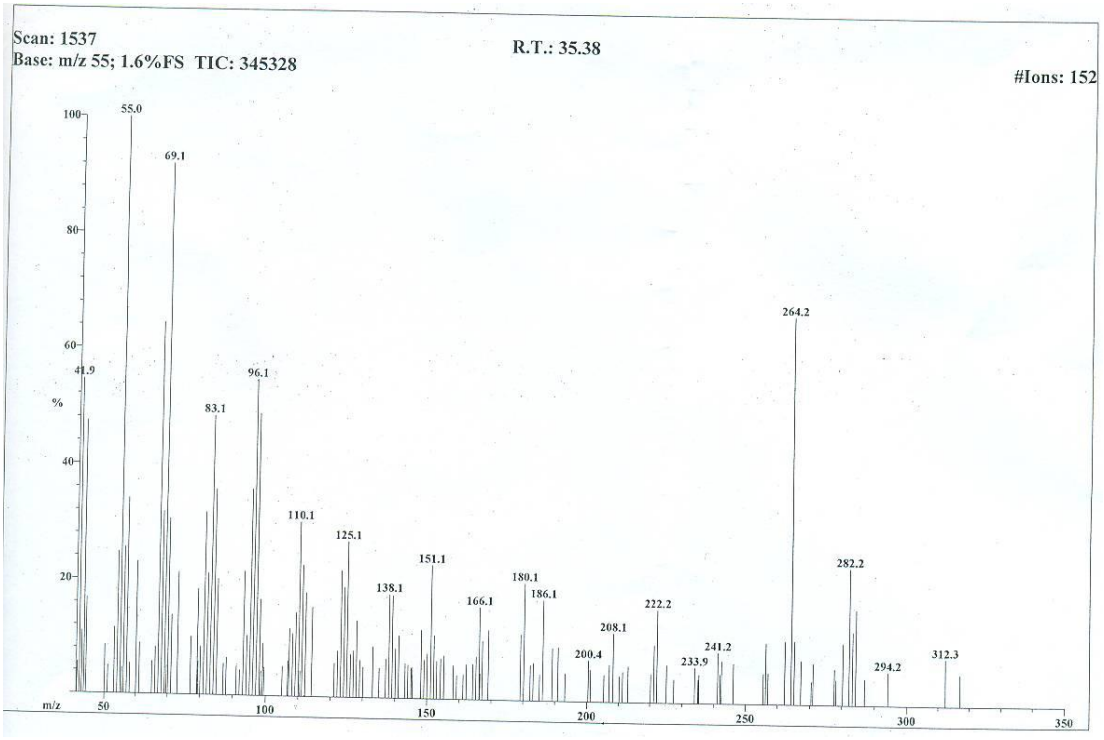


Figure 6. Chromatogram (GC-MS) of cell-free fluid from a co-culture of strains *P. aeruginosa* MY06, *P. aeruginosa* and *P. aeruginosa* SWD.

accumulative metabolic/enzymatic potentials of the three isolates.

The present results suggest that *Pseudomonas* has consistent genetic potential that immediately becomes responsive for detoxification of varied nature of pollutants such as detoxification potential of *Pseudomonas* for naphthalene, nylon oligomer, 4-chlorophenol and toluene, respectively (Guerin and Boyd, 1995; Prijambada et al., 1995; Mclaughlin et al., 2006; Duetz et al., 1996).

Yield of malaoxon by *Pseudomonas* sp. was paradoxically different from those of Bennett et al. (1984). However, other workers have reported the formation of malathion mono acid (MMA), malathion diacid (MDA) (Talebi and Shibamoto, 2007), malaoxon (Bavcon et al., 2003), diethyl thiosuccinate, O,O-dimethyl phosphorrothionic acid (Pehkonen and Zhang, 2002). Varying reports on the degradation products of malathion are suggestive for considering metabolic differences of the same species' isolates and their biodegradative potential for varying situations.

Numerous studies have suggested mechanism(s) involved in metabolism of malathion by *Pseudomonas*. Matsumura and Boush (1966) and Singh et al. (1989) isolated *Pseudomonas* sp. from a contaminated soil, which rapidly degrades malathion through carboxylesteratic hydrolysis as well as desmethylation processes.

Regarding the pathway of biodegradation of malathion, hydrolysis has been found as the most common one (Kim et al., 2005). A number of metabolites of malathion formed in plants, soil and water have been formed as a result of various chemical reactions like de-esterification, oxidation and hydrolysis (Kaur et al., 1997). Presently, the detection of malathion and its degradative metabolites is done routinely and successfully with the advanced techniques like GC/FID (Bavcon et al., 2003) as welll as GC/MS (Talebi and Shibamoto, 2007).

As far as the enzyme for malathion degradation is concerned, dicarboxylesterase has been found responsible both *in vivo* (Talebi and Shibamoto , 2007) and *in vitro* (Sun et al., 1992). The enzyme Carboxylesterase (Goda et al., 2010) hydrolase (OP hydrolase) has been isolated from several bacteria but with slower rate (Guha et al., 1997; Horne et al., 2002).

Further work on such lines may yield the identification of bacterial ecological relationship that might prove beneficial in designing biodetoxification model with complete removal of a pollutant with no consequent generation of metabolites that may bear toxicity even greater than the original substrate.

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Full Length Research Paper

Isolation, characterization and antimicrobial activity of a *Streptomyces* strain isolated from deteriorated wood

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Emergence of drug resistance among pathogenic bacteria to currently used antibiotics has made the search for novel bioactive compounds from natural and unexplored habitats a necessity. In this study, we reported the isolation, characterization and antimicrobial activity of an actinomycete strain isolated from deteriorated wood of an old house located in the Medina of Fez. The isolate, named H2, was identified by 16S rDNA sequencing and was shown to belong to the genus *Streptomyces*. The isolate was screened for antimicrobial activity against Gram positive bacteria, Gram negative bacteria, Mycobacteria, yeasts and fungi. Partial characterization of the active substance (resistance to proteinase K and heat) showed that it would be of non-protein nature. The kinetics of production of the active substance showed that the maximum production occurs between the 7th and 10th day of fermentation. In addition, organic extract of the isolate was able to release genomic DNA of *Staphylococcus aureus* suggesting that it acts probably on the bacterial cell wall. Thin layer chromatography (TLC) of the ethyl acetate extract followed by bioautography has allowed localizing the active substances. This will open the way to further investigations to demonstrate their potential importance in combating pathogenic bacteria.

Key words: Actinomycetes, *Streptomyces*, antimicrobial activity, molecular identification.

INTRODUCTION

Since the discovery of antibiotics, bacterial resistance to these chemicals has continued to evolve. Thus, we are witnessing more and more multiresistant bacteria that pose a serious public health problem. Therefore, novel antibiotics against drug resistant bacteria are urgently needed. Microbial natural products have been one of the major sources of novel drugs.

Among microorganisms, actinomycetes are the most economically and biotechnologically useful prokaryotes (Lam, 2006; Valli et al., 2012). They produce antibiotics and other industrially important secondary metabolites (Okami and Hotta, 1988; Koehn and Carter, 2005; Nermeen and Gehan, 2006; Kekuda et al., 2010; Naine et al., 2011).

From all the genera of actinomycetes, the genus *Streptomyces* is represented in nature by the largest number of species and varieties. These species can produce a large number of antibiotic and active secondary metabolites (Devi et al., 2006). In fact, 80% of the recognized antibiotics are sourced from this genus

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(Procópio et al., 2012), and many representatives of this group produce substances of high commercial value and are being extensively screened for novel bioactive compounds (Anderson and Wellington, 2001; Vijayakumar et al., 2007).

Exploring new habitats is one of the most promising ways to isolate new strains of actinomycetes endowed with antimicrobial activity (Zitouni et al., 2005; Khanna et al., 2011; Wadetwar and Patil, 2013).

Thus, we report here the isolation and molecular identification of a Streptomyces strain endowed with antibacterial activity. Partial characterization of the active substance as well as its mode of action is also reported.

MATERIALS AND METHODS

Sample collection

Pieces of deteriorated wood were taken from an old house, built 450 years ago, located in the former Derb Lamté in the Medina of Fez and were transported aseptically in sterile containers to the laboratory.

Isolation and culture conditions

Ten grams of wood samples were crushed and suspended in physiological water, then vigorously mixed for 2 h. The suspension was serially diluted up to 10^{-7}. An amount of 0.1 ml of each dilution was spread on the surface of the Actinomycetes Isolation Agar medium (AIA) (Thakur et al., 2007) and incubated at 30°C. After 2-4 weeks of incubation, the plates were examined for the presence of actinomycetes colony. The colonies of actinomycetes were recognized according to their macroscopic and microscopic characteristics (optical microscopy and Gram stain) then purified and conserved at 4°C for short periods and at -20°C in glycerol stock (20%, v/v) for a longer period. Twelve isolates showing different morphological characteristics were obtained. In this work, an isolate named H2 was chosen for the study of its antimicrobial activity.

Characterization of the isolate H2

The isolate H2 was tested for its ability to grow at pH 5 to 10 and at a temperature range of 25 to 42°C. Thus, actinomycetes cultures were spot inoculated onto plates of ISP2 (Shirling and Gottlieb, 1966) media to pH 5, 6, 7, 8, 9 and 10. The plates were checked for growth after seven days of incubation at 30°C. The same procedure was followed for the temperatures test except the use of ISP2 medium at pH 7.2. The temperatures tested were 25, 30, 37 and 42°C.

Actinomycetes synthesize and excrete dark pigments, melanin or melanoid, which are considered to be a useful criterion for taxonomical studies (Zonova, 1965; Arai and Mikami, 1972). Therefore the production of melanoid pigments was carried out on ISP6 and ISP7agar (Shirling and Gottlieb, 1966).

DNA extraction, PCR amplification and sequencing of 16S rDNA

The isolate H2 was grown for 4 days at 28°C with agitation in 10 ml of ISP2 medium. Biomass was harvested by centrifugation at 8000 rpm for 10 min and washed twice with sterile distilled water. The pellet was dispersed in 800 µl of the aqueous lysis solution (100 mM Tris-HCl, pH 7; 20 mM EDTA; 250 mM NaCl; 2% SDS; 1 mg/ml lysozyme). The suspension was incubated at 37°C for 60 min. About 10 µl of a proteinase K solution (20 mg/ml) was added, and the lysis solution was reincubated at 65°C for 60 min. The lysate was extracted with an equal volume of phenol and centrifuged at 7000 rpm for 10 min. The aqueous layer was reextracted with phenol (50-50%, v/v) and then by chloroform (50-50%, v/v). DNA was recovered from the aqueous phase by the addition of NaCl (150 mM final concentration) and two volumes of cool 95% (v/v) ethanol prior to centrifugation. The precipitated DNA was cleaned with 500 µl of 70% (v/v) ethanol, centrifuged at 7000 rpm for 10 min, resuspended in 20 µl of TE buffer (10 mM Tris-HCl, pH 7.4; 1 mM EDTA, pH 8), and stored at -20°C (Duraipandiyan et al., 2010).

The 16S rDNA gene of the isolate H2 was amplified by polymerase chain reaction (PCR) using primers fD1 (5' AGAGTTTGATCCTGGCTCAG 3') and Rs16 (5'TACGGCTACCTTGTTACGACTT 3') (Weisberg et al., 1991). The same primers were then used separately in two sequencing reactions from the two ends of the amplified fragment (about 1.5 kbp). The two sequences obtained were compared for similarity with those contained in genomic database banks, using the NCBI BLAST (Altschul et al., 1997).

Antimicrobial activity according to the culture medium

The actinomycete isolate was sown in scratches tightened in different agar media GLM (Kitouni et al., 2005), GYEA (Athalye et al., 1985), ISP2 (Shirling and Gottlieb, 1966), Bennett (Badji et al., 2005) and ISP1 (Shirling and Gottlieb, 1966) to show which medium stimulates maximum antimicrobial activity. After incubation for 7 days at 30°C, agar cylinders were then taken with hollow punch (Shomura et al., 1979; Saadoun and Al moumani, 1997; Petrosyan et al., 2003) and placed on Mueller Hinton agar plates, previously seeded with the test microorganism (10^5-10^6 CFU/ml). Plates were kept at 4°C for 2 h, and then incubated at 30°C for yeasts and at 37°C for bacteria and observed for antibiosis after 24 to 48 h. The bacteria and yeasts used as target were *Escherichia coli* DH5α, *E. coli* CIP 7624, *Staphylococcus aureus* CIP 53154**,** *Mycobacterium smegmatis* MC^2 155, *Mycobacterium aurum* A^+, *Bacillus subtilis* CIP 5262, *Bacillus cereus* CIP 14579, *Pseudomonas aeruginosa* 27853, *P. aeruginosa* A22, *Candida albicans* and *Candida tropicalis*.

Screening of antifungal activity

Antifungal activity was evaluated on ISP2 medium by the double layer method against a strain of *Aspergillus niger*. The isolate H2 was sown by touch in the center of Petri dish and incubated at 30°C during 7 days. The culture was covered by 8 ml of ISP2 medium containing 10 g of agar already sowed by *A. niger*. The diameter of inhibition was determined after 48 h of incubation at 30°C (Boughachiche et al., 2005).

Extraction of antimicrobial metabolites

The isolate of actinomycete was cultivated in ISP2 broth medium in 500 ml Erlenmeyer flask containing 100 ml of medium. The culture was incubated at 30°C for 7 days under constant agitation of 250 rpm. The production medium was centrifuged for 20 min at 8000 rpm to remove the mycelium. Ethyl acetate was added to the supernatant in the ratio 1:1 (v/v) and shaken vigorously for 2 h at room temperature. The organic extract was evaporated to dryness using a Rotavapor (Zitouni et al., 2005). The resulting dry extract

was recuperated in 1 ml of methanol and subjected to biological assay (disk of 6 mm in diameter) against *S. aureus*. The control corresponded to a disc containing an identical volume of methanol. The experiment was repeated twice. The dry extract was recovered in 1 ml of physiological water (NaCl 0.9%) instead of methanol to evaluate its effect on *S. aureus* cell wall (see below "extraction of staphylococcal DNA by the organic extract of the isolate H2").

Kinetics of antimicrobial activity of the isolate

Kinetics of antimicrobial activity was assessed on ISP2 broth. Pre-culture of the isolate H2 was carried out in 250 ml Erlenmeyer flask containing 50 ml of the ISP2 medium. After incubation for 48 h at 30°C under constant agitation at 250 rpm, the flask was homogenized and 5 ml of pre-culture was used to inoculate a 500 ml flask containing 100 ml of ISP2 medium. The flask was then incubited under the same conditions as above (Boudjella et al., 2006). The activity against *S. aureus* was regularly recorded each day by the agar diffusion method (well technique). The experiment was repeated twice.

Partial characterization of the antimicrobial products

The sensitivity to heat was examined by boiling the supernatant of the isolate H2 to 60, 80 and 100°C for 15 min then the treated supernatant was tested against *S. aureus* to determine its antibacterial activity by the well method. After incubation for 24 h at 37°C, the inhibition zone was measured. In addition, the sensitivity of organic extract to proteinase K was tested as follow: 40 µl of a Proteinase K solution (1 mg/ml) was mixed with 100 µl of the organic extract (using the protocol described previously) and incubated for 3 h at 37°C (Wu et al., 2005). Then, the effect of the Proteinase K treated extract was tested against *S. aureus* using the well method. The control was a solution of Proteinase K at the same concentration (the experiment was repeated twice).

Extraction of staphylococcal DNA by the organic extract of the isolate H2

A volume of 1 ml of an overnight culture of *S. aureus* was centrifuged at 5000 rpm during 5 min. The bacterial pellet was re-suspended in 360 µl of the organic extract recuperated in 1 ml of physiological water. This bacterial suspension was incubated at 37°C during 5 min and then centrifuged at 5000 rpm for 5 min. DNA was precipitated with two volumes of cool 95% (v/v) ethanol and 40 µl of NaCl 5 M. After incubation at -20°C during 30 min and centrifugation at 10000 rpm for 20 min, the pellet was dried and re-suspended in 20 µl of sterile distilled water. The control used corresponded to the same protocol as above, except for the use of 360 µl of physiological water instead of the organic extract (Hassi et al., 2007).

The genomic DNA obtained was detected by electrophoresis on 1% of agarose gel in a TAE buffer (Tris base 242 g; glacial acetic acid 57.1 ml; 0.5 M EDTA pH 8.0; H_2O 1000 ml) and was visualized by ultraviolet (UV) fluorescence after ethidium bromide staining. The experiment was repeated three times.

Thin layer chromatography

Ethyl acetate extract was used for primary analysis of the antibacterial substances. It was performed by thin layered chromatography (TLC) on silica gel slides by using chloroform-methanol (9:1, v/v) as solvent system. The chromatogram was observed under UV light. The experiment was repeated three times.

Bioautography

TLC slide was dried and was put in empty sterile Petri plate, in which 15 ml of sterile, LB agar seeded with *S. aureus* was poured. LB agar plate was incubated at 37°C for 24 h. After incubation, zone of inhibition around the spot was observed. The sterile zone on the media proved the presence of active antibacterial components in the studied samples (Holt, 1994). The Rf values of antibacterial compounds were determined.

In order to confirm the results from this experiment, the silica gel around the inhibition zone was eluted using ethyl acetate. After evaporation, the product was recuperated in sterile distilled water and 20 µl was spotted onto a sterile 6 mm diameter paper disc placed at the center of LB agar plate previously innoculated with a liquid culture of *S. aureus.* The experiment was repeated twice.

RESULTS AND DISCUSSION

Isolation of actinomycetes

In this study, an actinomycete isolate named H2 was isolated from deteriorated wood in an old house built 450 years ago located in the former Derb Lamté in the Medina of Fez. The isolate was Gram positive and formed colored tough and filamentous colonies that were hard to pick from the culture media as a characteristic of actinomycetes. It also produced colored pigments (purple), which were secreted into the culture media.

Characterization of the isolate

The growth of the isolate H2 was tested on ISP2 media at different pH and temperatures. We found that the isolate grew at pH and temperatures ranged from 5 to 10 and from 25 to 37°C, respectively.

For most actinomycetes, the optimum growth temperature is 23-37°C (Breidt et al., 1995; Stal and Moezelaar, 1997; Spyropoulou et al., 2001; Chen et al., 2003) whereas Goodfellow and Williams (1983) reported that most of the actinomycetes behave as mesophiles with an optimum growth at 30°C. There are also thermotolerant and thermophilic actinomycetes (Xu et al., 1998).

Streptomycetes are known to prefer neutral to alkaline environmental pH, the optimal growth pH range being 6.5 to 8.0 (Kutzner, 1986; Locci, 1989). However, acidophilic and alkalophilic streptomycetes have also been found (Kontro et al., 2005).

Melanin production was observed on the ISP6 medium but not on the ISP7 medium. This result is comparable with Antonova-Nikolova et al., (2006-2007) who found that *Streptomyces* sp. strain 34-1 synthesizes melanin on the ISP6 medium only and does not possess tyrosinase activity.

Some actinomycetes are capable of producing dark-brown substances in the culture media, generally referred to as melanin or melanoid pigments. Melanins are negatively charged composed of multi-functional polymers and polyphenolic compounds that are produced by various

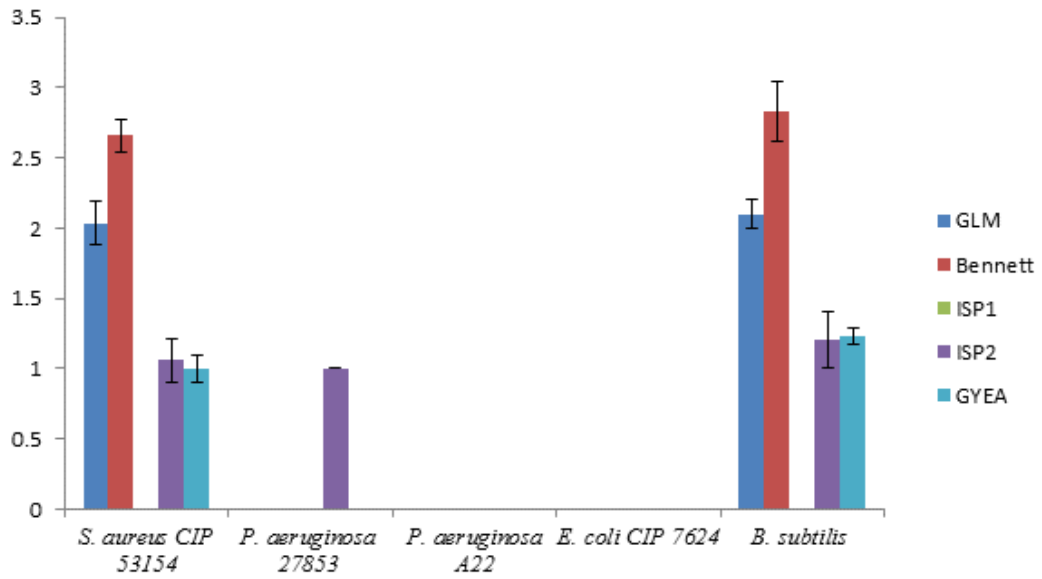

Figure 1. Antimicrobial activity of the isolate H2 on different agar media.

microorganisms by fermentative oxidation. They have also the radioprotective and antioxidant properties that can effectively protect the living organisms from ultra-violet radiation (Dastager et al., 2006). In addition they are used in medicine, pharmacology and cosmetics preparations (Dastager et al., 2006). Vasanthabharathi et al. (2011) found that the melanin pigment obtained from marine *Streptomyces* had antibacterial activity whereas Ali et al. (2011) found that pigment (melanin) produced by *Streptomyces virginiae* had antimicrobial activity against *A. niger*.

Molecular identification of the isolate H2

In this study, universal primers were used for amplification and sequencing the gene 16S rDNA of the isolate H2. The lengths of the 16S rDNA sequences analyzed were 540 and 550 bp for the primers fD1 and Rs16, respectively. Their analysis in comparison with the sequences available in Gen Bank, EMBL, DDBJ and PDB databases showed that the isolate H2 was closely related to *Streptomyces* sp. with 98 and 100% similarity for sequences obtained by fD1 and Rs16, respectively. This result is consistent with literature; in fact, the universal primers seem to be sufficient for identifying the genus but not the species (Boudemagh et al., 2005; Jeffrey, 2008; Arasu et al., 2008). Therefore, it is necessary to consider both the genetic and phenotypic aspects to identify the actinomycetes (Goodfellow et al., 2004). Other technics were used such as PCR-RFLP gene encoding 65-kilodalton heat shock protein (Steingrube et al., 1997), repetitive intergenic DNA sequences (Sadowsky et al., 1996) and sequencing of the rpoB gene encoding the β subunit of RNA polymerase (Kim et al., 2004).

Antimicrobial activity according to the culture medium

Antibiotic production by actinomycetes is dependent on the composition of the medium (Singh et al., 2009) and especially on carbon and nitrogen sources (Iwai and Omura, 1982).

To determine the best production medium of antimicrobial substances by the isolate H2, the following culture medium were used: GLM, GYEA, ISP2, ISP1 and Bennett (Figure 1). First, we noticed that the spectrum of activity was different depending on the culture medium used. ISP1 seems to be a poor production medium since no activity was observed on it. Badji et al. (2005) found that GYEA and ISP1 media enabled satisfactory production in comparison with Bennett, ISP2 and nutrient agar whereas Cheraiti and Gacemi kirane (2012) showed that the activity of strain Act sp5 was very important on GYEA medium as compared to ISP1, Bennett and ISP2.

No activity was observed on all media for strains *E. coli* CIP 7624 and *P. aeruginosa* A22 indicating that these strains might be resistant to the substances produced by the isolate H2. These substances were active on *S. aureus* CIP 53154 and *B. subtilis* CIP 5262 on GLM, Bennett, ISP2 and GYEA. It is interesting to note that *P. aeruginosa* 27853 was sensitive only on ISP2 medium. This medium is recognized in the literature as a good production medium (Badji et al., 2006, 2007). So, in the following, ISP2 medium were used as the production medium.

Screening of antimicrobial activity of the isolate H2 on ISP2 medium

The isolate H2 was active against Gram positive bacteria

Table 1. Antibacterial activity of the isolate H2.

Test isolate*								
EcDH5α	**Ec CIP**	**Bs**	**Bc**	**Pa A22**	**Pa**	**Sa**	**Ms**	**Ma**
-	-	+	+	-	+	+	+	+

+ Inhibition; - no inhibition. *EcDH5α, *E. coli* DH5α; Ec CIP, *E. coli* CIP 7624; Bs, *B. subtilis* CIP 5262 ; Bc, *B. cereus* CIP 14579 ; Pa A22, *P. aeruginosa A22;* Pa, *P. aeruginosa 27853*; Sa, *S. aureus* CIP 53154; Ms, *M. smegmatis* MC^2 155 and Ma, *M. aurum* A^+

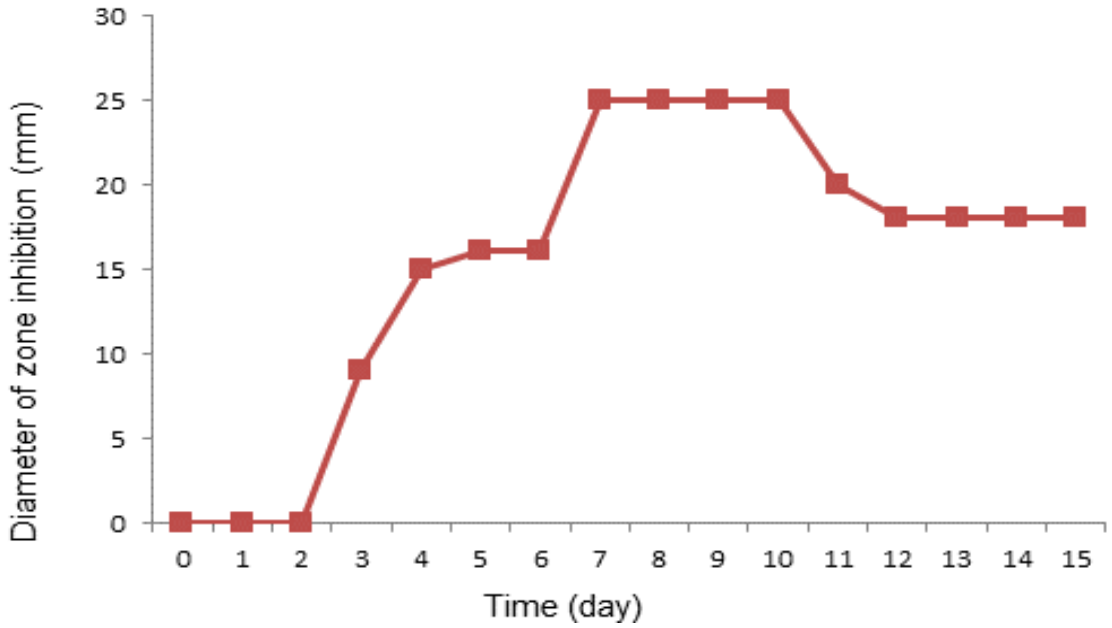

Figure 2. Kinetics of antimicrobial activity of the isolate H2 against *S. aureus.*

(*B. subtilis* CIP 5262, *B. cereus* CIP 14579 and *S. aureus* CIP 53154), Gram negative bacteria (*P. aeruginosa* 27853), Mycobacteria (*M. smegmatis* MC^2 155 and *M. aurum* A^+) whereas Gram negative bacterium *E. coli* DH5α, *E. coli* CIP 7624 and *P. aeruginosa* A22 were resistant (Table 1).

Furthermore, no activity was observed on yeasts and fungi tested in this study suggesting that the isolate H2 did not possess an antifungal activity.

Kinetics of antimicrobial activity of the isolate H2

The kinetics of production was carried out on ISP2 broth and the zones of inhibition against *S. aureus* were measured by the agar diffusion method (Figure 2). The antimicrobial activity was detected after 2 days of incubation and the maximum production was detected between the 7th and 10th day of fermentation. This result is consistent with the literature. In fact, it is now established that the optimum production of antimicrobial substances by actinomycetes coincides with the phase of differentiation. Kojiri et al. (1992) reported that the opti-mum production by *Streptomyces* sp. was reached at 6th day of incubation whereas Bouras et al. (2013) reported that the time of maximal antimicrobial activity by *Streptomyces* sp. PP14 was obtained always after 7, 8 or 9 day of fermentation.

Partial characterization of the antimicrobial product

The active substance of the isolate H2 inhibited *S. aureus* growth creating an inhibition zone around the wells. This activity was not eliminated upon treatment by Proteinase K (Table 2) or temperature (60, 80 and 100°C) (Table 3). It is concluded that the active substance might be of non protein nature.

Extraction of staphylococcal DNA by organic extract of the isolate H2

The active substance from the isolate H2 was extracted

Table 2. Sensitivity of the active substance from the isolate H2 to Proteinase K.

	Inhibition diameter against *S. aureus* (cm)		
	Without treatment with Proteinase K	**After treatment with Proteinase K**	**Control**
Organic extract of the isolate H2	3 ± 0.28	2.85 ± 0.07	0

The control used was a solution of Proteinase K. Values are means of two replicates ± standard deviation.

Table 3. Sensitivity of the active substance from the isolate H2 to temperature.

	Inhibition diameter against *S. aureus* (cm)			
	Not subjected to heat treatment	**After heat treatment**		
		60°C	**80°C**	**100°C**
Supernatant of the isolate H2	3 ± 0.14	3 ± 0.00	3 ± 0.14	2,6 ± 0.00

Values are means of two replicates ± standard deviation.

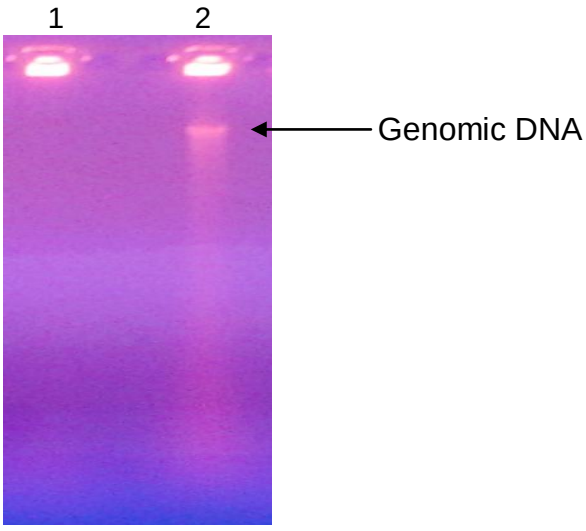

Figure 3. 1% Agarose gel electrophoresis analysis of *S. aureus* genomic DNA extracted with the organic extract of the isolate H2. 1: Control corresponding to DNA extraction of *S. aureus* with physiological water. 2: DNA extraction of *S. aureus* with the organic extract of the isolate H2.

by ethyl acetate, recuperated in physiological water and then tested for its ability to extract genomic DNA from *S. aureus* (previously explained in methods). Figure 3 shows that genomic DNA was obtained when *S. aureus* was treated by the organic extract of the isolate H2 in comparison with the control where organic extract was replaced by physiological water. This extraction was carried out in the absence of conventional agents of lysis, such as lysozyme, SDS or proteinase K, suggesting that the active ingredient acts at the *S. aureus* wall level.

Other active substances produced by actinomycetes act on cell wall biosynthesis (Schneider et al., 2009; Hendlin et al., 1969; Krogstad et al.,1980; Singh et al., 2003).

More experiments are needed to characterize the structure of the active substance produced by the isolate H2 and to illustrate its effect on the bacterial cell wall of *S. aureus*. The effect might be also studied on other bacteria, mainly Gram negative ones. Thus, a protocol based on this substance could be developed and used in extraction of bacterial genomic DNA.

Thin layer chromatography and bioautography

TLC and bioautography are often used for preliminary characterization of antimicrobial substances from producing microorganisms' organic extract, especially determination of the position of the hot spot on TLC (Considinet and Mallette, 1965; Awais et al., 2007).

In this study, chloroform-methanol (9:1, v/v) was used as solvent system to study ethyl acetate extract of the isolate H2. TLC silica gel analysis showed three bands having respectively the following Rf values: B1=0.37; B2=0.6 and B3=0.7 (Figure 4a). Bioautography of the TLC slide against *S. aureus* resulted in the formation of inhibition zones around the components corresponding to B2 and B3 bands (Figure 4b). No inhibition zone was observed around the B1 band.

Silica gel around the three bands was separately eluated and evaporated. The resulting products were separately recuperated in 20 µl of sterile distilled water and tested using the disc method. The results obtained confirms that the active bands were B2 and B3 (Figure 5). Further work is needed to determine the chemical structure of the products contained in the bands 2 and 3. The spectrum of activity of each of the two products should be determined on different bacteria and fungi. It would also

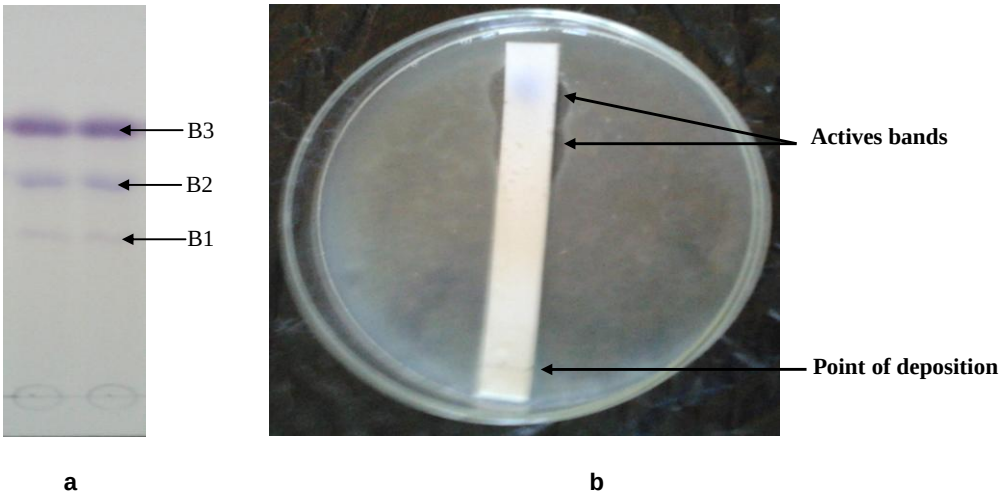

Figure 4. a) TLC analysis of the crude extract of H2 strain after migration; b) Bioautography of the H2 extract against *S. aureus*.

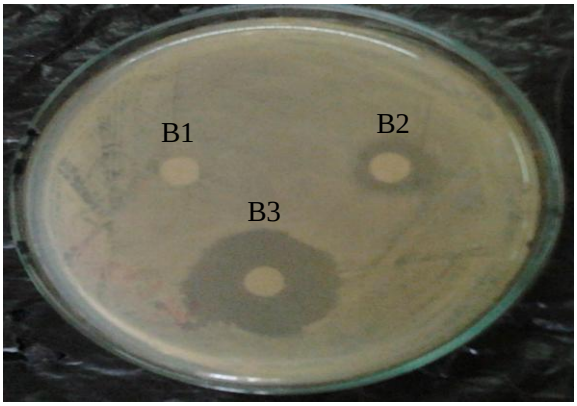

Figure 5. Effect of the three products (B1, B2 and B3) on *S. aureus* growth.

be interesting to see if the two products have a synergic effect or not.

In summary, a *Streptomyces* strain producing at least two antimicrobial substances was isolated from deteriorated wood. The activity of the isolate H2 against *S. aureus*, Mycobacteria and *P. aeruginosa* makes it more interesting to study because of the problems of resistance and multi-resistance encountered by these bacteria.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Selection and characterization of *Clostridium bifermentans* strains from natural environment capable of producing 1,3-propanediol under microaerophilic conditions

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In this work, we aimed to select from natural environment non-pathogenic strains of *Clostridium* spp. capable of producing 1,3-propanediol (1,3-PD). As a result we isolated 2256 of *Clostridium* spp., including 10 strains from the genus *Clostridium bifermentans*. It occurred that all isolates of this species were able to synthesis 1,3-PD on the level of ca. 10 g/L. Additionally, they synthetized metabolites in microaerophilic conditions, which is very profitable from the industrial point of view. We characterized morphological and physiological properties of all isolated strains of *C. bifermentans*. These tests demonstrated significant dissimilarity among all isolates of *C. bifermentans* species.

Key words: Aerotolerant, *Clostridium bifermentans*, glycerol fermentation, isolation, 1,3-propanediol.

INTRODUCTION

Conversion of renewable biomass to solvents, acids, and fuels is a good alternative to chemically synthesized products. Nowadays, there are numerous possibilities for replacing chemical techniques with biotechnological methods based on renewable resources (Willke and Vorlop, 2004). For this aim, microbial strains from the ATTC or other collections are often used. Indigenous bacteria in the natural environment, however, can produce a wide range of metabolites more efficiently (Lopez et al., 2004; Leja et al., 2011). An important group of industrially useful microorganisms is *Clostridium* spp. These species are able to metabolize an extremely wide range of organic molecules, among others carbohydrates, organic acids, alcohols, aromatic compounds, amino acids, amines, purines, and pyrimidine. They can be used in the production of solvents (butane, acetone, and ethanol) and acids (succinic, acetic, and butyric) (Ishii et al., 2000; Mitchell, 2001). Some *Clostridium* spp. such as

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Clostridium butyricum (Colin and Bories, 2000), *Clostridium pasteurianum* (Biebl et al., 1992; Dabrock et al., 1992), *Clostridium diolis*, *Clostridium acetobutylicum*, *Clostridium butylicum*, *Clostridium perfingens*, (Youngleson et al., 1998; Hao et al., 2008) are also able to biosynthesis 1,3-propanediol (1,3-PD). However, in the literature there is no information about the production of 1,3-PD by *Clostridium bifermentans*. In addition, there is no information that 1,3-PD might also be produced from glycerol by *Clostridium* strains in microaerophilic conditions. Kawasaki et al. (1998) stated that *C. butyricum* possesses the ability to consume oxygen and the ability to grow when oxygen is present but there is no information whatsoever as to the metabolite production in oxygen conditions.

The 1,3-PD, a typical product of glycerol fermentation, is one of the most interesting raw materials for chemical industries as it demonstrates a wide range of use in different fields, for example, it is a valuable chemical intermediate applied in organic synthesis. It is also used as a monomer for the production of biodegradable polymers (polyesters, polyether, polyurethanes, etc.), cosmetics, lubricants, medicines, and as an intermediate for the synthesis of heterocyclic compounds (Regan and Crawford, 1994; Menzel et al., 1997; Katrlík et al., 2007; Biebl et al., 1999; Leja et al., 2011). Recently, 1,3-PD has also been used as a monomer to synthesize a new type of polyester - polytrimethylene terephthalate (Biebl et al., 1999; Zeng and Biebl, 2002; Liu et al., 2007; Zhang et al., 2007).

In our work, we selected from the natural environment aerotolerant *C. bifermentans* strains that is able to produce 1,3-PD. *C. bifermentans* which was first isolated by Tissier and Martelly in 1902 (Brooks and Epps, 1958). The original name of this strain was *Bacillus bifermentans sporogenes* (Tissier and Martelly, 1902) and later it was re-named as *B. bifermentans* (Weinberg and Seguin, 1918), in accordance with the principle of binominal nomenclature. *C. bifermentans* is described as an anaerobe, catalase-negative, Gram-positive, endospore forming and motile strain (Regan and Crawford, 1994). However, according to Brooks and Epps (1958) *C. bifermentans* is non-motile, and only some young cultures are motile. These incoherent data probably resulted from a huge bio-diversity of the genus of *Clostridium*. Strains which were isolated and characterized during the investigation described above are a good evidence of this hypothesis.

MATERIALS AND METHODS

Microorganisms, growth media, and cultivation conditions

Three thousand eight hundred and twenty-seven (3827) samples from excrements of animals and composts, and silages, samples from biogas works, soils, active sludge, rivers' sludge and wastes from food industry were collected in the region of the Wielkopolska District, Poland, during the period of January - July 2011. These samples were collected in sterile plastic jars and stored in refrigerator until experimentations (no longer than one month).

Samples were pre-cultured, in 10 mL test tubes, on modified PY medium (peptone-yeast medium) according to Biebl and Spöer (2002). This medium consisted of (g/L): BactoPeptone 10; yeast extract 10; glycerol 50; $CaCl_2$, $MgSO_4 \times 7H_2O$ 0.96; K_2HPO_4 2; $NaHCO_3$ 20, and NaCl 4. The pre-cultivation step was conducted in anaerostats in two variants - under microaerophilic condition (95% CO_2 and 5% O_2) at 30°C for 7 days, according to the method described in Myszka et al. (2012), and in anaerobic conditions with CO_2 flux only. The microanaerobic conditions in flask were maintained by CampyGen Kit (Oxoid, UK), while anaerobic by Gas Generating Kit (Oxoid, UK). After incubation, the samples were pasteurized (80°C, 10 min.), diluted with sterile solution of 0.85% sodium chloride and then spread onto TSC agar plates (Tryptose Sulfite Cycloserine Agar Base) (BD, USA). The plates were incubated for 24 h at temperature of 30°C. Isolated colonies (5 colonies per 1 plate) were screened on the basis of their morphological character (black colonies or black ones with a 2-4 mm opaque white zone surrounding the colonies as a result of lecithinase activity). To make pure culture and maintain culture conditions for the bacteria, screened colonies were transferred on both TSC agar plates (BD, USA) and RCM broth (Reinforced Clostridial Medium) (BD, USA). Isolated bacterial strains were allowed to grow in modified PY medium for 7 days at 30°C in 2 mL eppendorf tubes. After incubation, the broths were centrifuged (3000 rpm, 10 min). The cell free supernatants were collected and used for estimation of 1,3-PD production. For this purpose high performance liquid chromatography (HPLC) analyses were done. Hewlett Packard system consisted of auto sampler and pump, and a refractive index detector was carried out. The analyses were performed isocratically at a flow rate 0.6 ml/min at 65°C, on column Aminex HPX-87H 300x7.8 (BIO-RAD). 0.5 mM H_2SO_4 was used as a mobile phase. Standards were applied to identify peaks in chromatograms, and peak areas were used to determine samples concentration. This procedure was conducted by computer integration (ChemStation, Agilent) operated in the mode of external standards. The chromatography analyses were done duplicate for each sample. In order to identify 10 selected isolated strains with the highest level of 1,3-PD synthesis, the sequencing and phylogenetic analyses were done. Next, all strains were characterized by physiological and biochemical methods.

Sequencing and phylogenetic analysis

Total DNA from bacteria was extracted with the use of Genomic Mini AX Bacteria Kit (A&A Biotechnology, Gdańsk, Poland) after an initial incubation in 50 mg/mL lysozyme (Sigma) for 1 h at 37ºC. Sequences encoding small subunit of rRNA were amplified in PCR reaction using primers SDBact0008aS20 and SUniv1492bA21 (41). PCR products were purified using Clean-up Kit (A&A Biotechnology, Gdańsk, Poland) and sequenced at Genomed Co. Warsaw, Poland with primers used for PCR, and additionally for inner sequence with GTGCCAGCMGCCGCCCTAA primer. PCR was performed in the total volume of 100 μL containing 1xPCR buffer (10 mM KCl, 10 mM $(NH4)_2SO_4$, 20 mM Tris-HCl (pH 8.5), 2 mM $MgSO_4$, 0,1% Triton X-100), 25 ng DNA template, 0.44 μM concentration of each primer, 200 μM dNTP, 2,5U Run DNA Polymerase (A&A Biotechnology). PCR was carried out in the Biometra T Gradient thermocycler. The amplification of 16S rDNA consisted of 15 cycles: 1 min denaturation step at 94ºC, 1 min annealing step at 48ºC, and 2 min extension at 72ºC. The size of the amplified fragments was equal 1 500 base pairs. The sequences obtained were

arranged into contigs and identified in BLAST service of the GenBank database (Altschul et al., 1990; Suau et al., 1999).

Morphological tests

All the strains were grown on agar plates containing TSC medium with 1.5% of agar (BD, US). To describe the morphology of the colonies, inoculated plates were incubated for 24 h.

Motility test

The motility test medium was used to demonstrate whether or not the cells can swim in a semisolid medium (PY with 1% of agar). A semisolid medium was inoculated with the bacteria in a straight-line stab with a needle. After incubation bacterial growth can be observed away from the line of the stab, which is evidence of the fact the bacteria were able to swim through the medium.

Urease production test

To check the urease production ability, the cultures were grown for 24 and 48 h at 37°C in 20 ml amounts of PY medium. A few strains from each group were also grown in 100 ml amounts of a similar medium and tested daily for 4 days. The following reagents were used: KH_2PO_4 (0.1 g), K_2HPO_4 (0.1 g), NaCl (0.5 g), urea (2.0 g)., 95% (v/v) ethanol in water (1.0 ml), distilled water (100 ml), Universal Indicator (5.0 ml), sufficient 0.1_N -HCl give an orange colour (about pH 6.0). A 2.5 ml sample of the culture under testing was transferred to a test tube (80 mm × 8.0 mm) and centrifuged. The supernatant fluid was discarded and the sediment washed once in 1.0 (v/w) saline and finally resuspended in 1.0 ml distilled water. One milliliter of urease reagent was added and, after thorough mixing, the test was incubated at 37°C (8).

Indole production test

Twenty four hour cultures in peptone broth were examined for indole production by means of Ehrlich's reagent and by the vanillin test (Sprayr, 1936).

Sugar assimilation and fermentation tests

In sugar assimilation tests, glycerol was replaced in PY medium by such saccharides as glucose, fructose, arabinose, maltose, mannitol, glycerol, sorbitol, rhamnose, and xylose. Additionally, phenol red was used (1 mg/ml) as an indicator. The pH of the medium was adjusted to 8.6. The medium was dispensed into 10 mL tubes, sterilized by autoclaving, and then bacteria were introduced to the medium (the size of bacterial inoculum was equal 10% v/v). The pure culture of the isolates was incubated at 36°C for 24 h in 10mL test tubes, while the result was indicated by a change of color from red to yellow (Shrestha and Sharma, 1995). Control tubes were used in each set to monitor contamination.

In sugar fermentation tests, glucose, fructose, arabinose, maltose, mannose, mannitol, glycerol, sorbitol, rhamnose, and xylose were added to the PY medium. To analyze their fermentation, the technique of high liquid chromatography was applied. The analysis were performed isocratically at a flow rate 0,6 ml/min. at 65°C, on column Aminex HPX-87H 300x7.8 (BIO-RAD). 0.5 mN H_2SO_4 as a mobile phase was used. Standards were applied to identify peaks in chromatograms, and peak areas were used to determine samples concentration. It was conducted by computer integration (Chem-Station, Agilent) operated in the mode of external standards. As a control probe, the PY medium with glycerol was used. All experiments were done in duplicate.

Antibiotic sensitivity test

To characterize antibiotic sensitivities of the clostridial isolates they were tested against batteries of both traditional and non-traditional anti-clostridial antibiotics, using a rotary test method. Bacteria strains were spread onto plate dishes and the TSC medium was used. Then, the rotaries with different antibiotics were put onto plate dishes. After 24 h, in case of the strains which were sensitive to the antibiotic, a bright zone around the rotary was observed. All tests were done in duplicate.

The oxidoreduction potential of isolated strains

In this experiment the flow cytometry technique was applied. The redox potential in bacteria cells cultivated without oxygen was compared with the potential in bacteria cells cultivated with a small amount of oxygen (5%). The high value of the redox potential means that the cells were alive and the metabolic activity of these cells was high..

The samples analyzed comprised growth cultures of 10 *C. bifermentans* strains. Bacteria were cultivating for 24 h in microaerophilic (5% O_2 and 95% CO_2) and in strictly anaerobic conditions. Flow cytometric analysis of microbial cells' vitality and metabolic activity with redox potential as a relevant parameter were evaluated using BacLight Redox Sensor Green Vitality Kit from Invitrogen Company. For analysis, 1 ml of each culture was collected by centrifugation, resuspended in 1% PBS and prepared according to manufacturer's manual. Prior to the analysis, an optimization step was involved in order to assess the reagents appropriate staining concentrations. Sample analysis was performed using BD FACS Aria™III (Becton Dickinson) flow cytometer (cell sorter), equipped with four lasers (375, 405, 488 and 633 nm), 11 fluorescence detectors, forward scatter (FSC) and side scatter (SSC) detectors. The primary sample line was fitted in initial 50 µm-pore-size filter, preventing flow arrest in case of samples including particles capable to block the light of sample line or a nozzle. The instrument setup (optical alignment), stability and performance tests were made using CST system (Cytometer Setup and Tracking) from Becton Dickinson company. FACSFlow solution (Becton Dickinson) was used as sheath fluid. The configuration of the flow cytometer was as follows: 70 µm nozzle and 70 psi sheath fluid pressure. The cells were characterized by two non-fluorescent parameters: forward scatter (FSC) and side scatter (SSC), and two fluorescent parameters: green fluorescence (FL1) from RedoxSensor™ Green reagent collected using 530/30 band pass filter and red fluorescence (FL2) from propidium iodide (PI) reagent collected using 616/23 band pass filter. For excitation of both fluorescent reagents, 488 nm laser was employed. The flow cytometry analyses were performed by using logarithmic gains and specific detectors settings. The threshold was set on the FSC signal. Data were acquired in a four-decade logarithmic scale as area signals (FSC-A, SSC-A, FL1-A and FL2-A) and analyzed with FACS DIVA software (Becton Dickinson). The analysis of fluorescence signals from both fluorochromes preceded doublets discrimination procedure with the use of height versus width scatter signals measurement, in order to discriminate single events from conglomerates. The populations were then defined by gating in the dot plots of green fluorescence (FL1) versus red fluorescence (FL2). Each sample was analyzed in triplicate. The estimation of

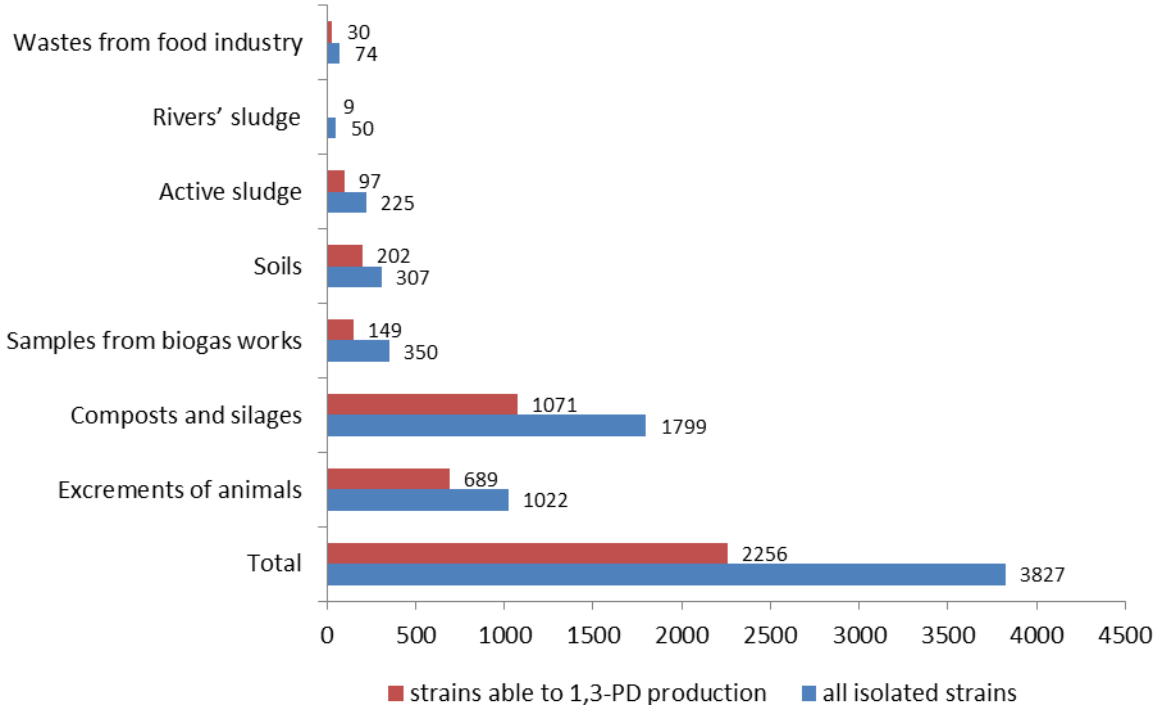

Figure 1. Samples processed and obtained isolates.

cells redox potential was performed using medians of green fluorescence (FL1) signals of gated populations defined on bivariate dot plot (FL1 vs. FL2).

RESULTS

Isolation of Clostridium strains and phylogenetic identification

In our study, PY medium proposed by Biebl and Spöer (2002) was tested for its ability to isolate bacteria of the *Clostridium* genus from natural samples. The aim of this work was to isolate bacteria strains that is able to produce 1,3-PD. Accordingly, *Clostridium* spp was isolated from all tested natural samples (Figure 1). The highest number of isolates of the genus of *Clostridium* was obtained from excrements of animals (1,022 isolates) and composts or silages (1,799 isolates). In addition, in our study all bacterial strains were tested for their ability to grow with glycerol in the medium and to converse this substrate to 1,3-PD. Of the total number of 3,827 isolates tested nearly 56% (2,256 isolates) fermented glycerol to 1,3-PD. Excrements of animals as well as compost and silages contained the highest numbers of isolates able to produce 1,3-PD (Figure 1). The 12 strains obtained from soil and composts, which synthetized most 1,3-PD, were identified by sequencing as C. *bifermentans* and *C. butyricum*. Interestingly, the glycerol fermentation by *C. bifermentans* species has not been investigated yet. The results of *C. bifermentans* identification are presented in Table 1.

The ability of production of 1,3-PD by isolated strains

In our experiments we investigated the ability to produce 1,3-PD, in both anaerobic and microaerophilic conditions, by 10 strains identified previously as a *C. bifermentans*. The yield of 1,3-PD production in anaerobic and microaerophilic condition (95% $C0_2$ and 5% O_2) via glycerol fermentation by examined strains of *C. bifermentans* is presented in Figure 2. The metabolite profile was the same independent of cultivation conditions. The range of 1,3-PD production was between 9.78 and 14.89 [g/L] ($Y_{p/s}$=0.20-0.30; P_a=0.06-0.09). During these fermentations other metabolites were also obtained. The amount of acids synthetized from glycerol fermentation by selected microflora is presented in Table 2. The metabolic pathway in all 10 strains was similar but not the same (efor example, strains *C. bifermentans* 371, 540, 541 were not able to synthetize ethanol, *C. bifermentans* 549 did not produce succinic acid, and *C. bifermentans* 546 was not able to synthesis formic acid). As a reference culture *C. bifermentans* (ATCC 638T) obtained from the American Type Culture Collection was used. The reference cultures were also grown on the PY medium in the same microaerophilic and anaerobic conditions. The

Table 1. Strains identification.

Strain number	Isolation source	Homology (%)	Bacteria species	NCBI number
Strain 371	silage	98	*C. bifermentans*	JQ407555
Strain 374	silage	99	*C. bifermentans*	JQ407557
Strain 376	silage	98	*C. bifermentans*	JQ407556
Strain 535	forest soil	98	*C. bifermentans*	JQ407583
Strain 537	forest soil	98	*C. bifermentans*	JQ407582
Strain 540	forest soil	98	*C. bifermentans*	JQ407563
Strain 541	forest soil	99	*C. bifermentans*	JQ407564
Strain 543	forest soil	99	*C. bifermentans*	JQ407565
Strain 546	forest soil	99	*C. bifermentans*	JQ407566
Strain 549	forest soil	99	*C. bifermentans*	under deposition

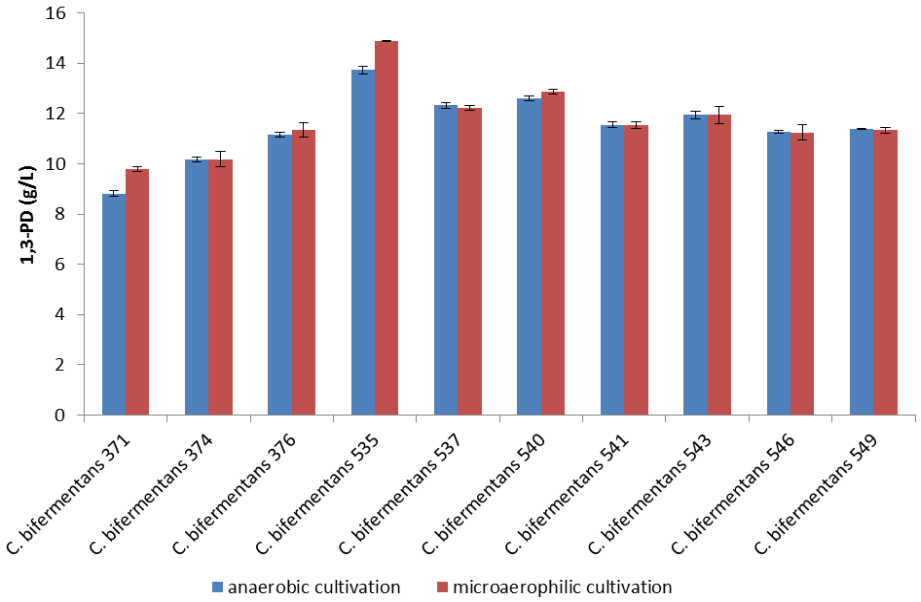

Figure 2. Biosynthesis of 1,3-PD production by isolated strains of *C. bifermentans*.

C. bifermentans ATTC 683 strains produces 13.35 [g/L] of 1,3-PD from 50 [g/L] of glycerol (our data, unpublished). This result was comparable with the results obtained in production with our 10 strains isolated from the natural environment. It confirmed the fact that *C. bifermentans* is able to produce 1,3-PD in anaerobic and microaerophilic conditions. Additionally, we decided to check the ability of other collections of strains such as *C. diolis* DSMZ 15410, *C. butyricum* DSMZ 10702, and *C. pasteurianum* ATCC 6013 to 1,3-PD production in microaerophilic conditions. It occurred that in the presence of oxygen *C. diolis* and *C. butyricum* are not able to produce 1,3-PD. *C. pasteurianum* produced only 1.44 [g/L] of 1,3-PD from 50 [g/L] of glycerol. All the three above mentioned strains are known as good 1,3-PD producers in anaerobe but in the presence of oxygen they inhibited metabolic activity of these strains. Table 2 shows isolation sources of each *C. bifermentans* strain. The best isolation source for the 1.3-PD producers were silage and forest soil. Strains obtained from the forest soil are able to produce 1.3-PD on the higher level than the strains obtained from the silage. The best 1.3-PD producer obtained from the soil formed 14.89 [g/L] in the PY medium supplemented with 50 [g/L] of glycerol (*C. bifermentans* 535).

Oxidoreductive potential

During our work tolerance of investigated strains on oxygen was observed. It is noteworthy that in the literature there is information that *C. bifermentans* is an obligate

Table 2. Profile of metabolites of glycerol fermentation by selected *C. bifermentans* strains

Strain number	isolation source	LA (g/L)		FA (g/L)		AA (g/L)		SA (g/L)		BA (g/L)		Et (g/L)	
		AC	MC	AC	MC	AC	MC	AC	MC	AC	MC	AC	MC
371	silage	8.21 ± 0.50	8.19 ± 0.08	1.91 ± 0.11	1.94 ± 0.08	3.92 ± 0.05	3.81 ± 0.15	5.98 ± 0.06	6.20 ± 0.11	nd	nd	nd	nd
374	silage	8.64 ± 0.07	8.67 ± 0.05	2.36 ± 0.17	2.34 ± 0.25	3.72 ± 0.17	3.73 ± 0.15	0.11 ± 0.02	0.20 ± 0.03	nd	nd	0.23 ± 0.02	0.49 ± 0.02
376	silage	6.66 ± 0.02	6.66 ± 0.03	1.34 ± 0.13	1.36 ± 0.11	2.92 ± 0.21	3.04 ± 0.04	1.88 ± 0.08	1.73± 0.06	nd	nd	0.29 ± 0.01	0.44 ± 0.11
535	forest soil	6.53 ± 0.04	6.66 ± 0.014	1.72 ± 0.04	1.66 ± 0.07	2.12 ± 0.32	2.04 ± 0.02	1.02 ± 0.11	0.69 ± 0.05	nd	nd	0.88 ± 0.03	0.84 ± 0.01
537	forest soil	6.08 ± 0.08	6.16 ± 0.13	2.02 ± 0.11	1.94 ± 0.08	3.91 ± 0.03	3.80 ± 0.13	0.12 ± 0.09	0.22 ± 0.01	nd	nd	0.77 ± 0.02	1.00 ± 0.04
540	forest soil	11.08 ± 0.07	11.29 ± 0.06	1.63 ± 0.10	1.77 ± 0.09	2.13 ± 0.15	2.17 ± 0.14	0.14 ± 0.02	0.28 ± 0.07	nd	nd	nd	nd
541	forest soil	10.82 ± 0.12	10.96 ± 0.11	nd	nd	1.67 ± 0.23	1.67 ± 0.25	1.13 ± 0.01	1.03 ± 0.03	nd	nd	nd	nd
543	forest soil	11.32 ± 0.09	11.34 ± 0.18	2.31 ± 0.06	2.33 ± 0.16	5.18 ± 0.13	5.23 ± 0.21	0.79 ± 0.06	1.09 ± 0.01	nd	nd	1.76 ± 0.01	1.93 ± 0.14
546	forest soil	10.78 ± 0.10	10.80 ± 0.13	nd	nd	0.93 ± 0.03	1.00 ± 0.04	0.11 ± 0.02	0.13 ± 0.01	nd	nd	0.77 ± 0.01	0.52 ± 0.03
549	forest soil	7.18 ± 0.12	7.17 ± 0.21	1.74 ± 0.11	1.76 ± 0.05	2.76 ± 0.03	2.65 ± 0.07	nd	nd	nd	nd	1.55 ± 0.01	1.73 ± 0.08

nd- not detected, AC - anaerobic conditions, MC - microaerophilic conditions. LA - lactic acid; FA - formic acid; AA - acetic acid; SA - succinic acid; BA - butyric acid; Et - ethanol

anaerobe (Regan and Crawford, 1994). Thus, we decided to analyze the redox potential using the flow cytometry method. The aim of this experiment was to compare the metabolic activity of bacteria cultivated in the presence of small amount of oxygen and without it.

In order to assess the metabolic activity of bacterial strains analyzed, each sample was divided into three tubes, corresponding to unstained control, negative control and positive. Negative control comprised tubes with a CCCP reagent added, which acts as an electron transport chain uncoupler. After incubation, RedoxSensor™ Green reagent and propidium iodide were added to negative control and positive tubes. Thus the negative control represents a reference sample for normalization of green fluorescence signals from cells with stimulated reduction of redox potential (CCCP treated) and cells non-treated with an electron chain uncoupler, allowing us to estimate the metabolic activity of the cells. The difference in medians of green fluorescence signals corresponds to redox potential of the cells analyzed. This permits a strain comparison for selection of the ones with highest redox potential relevant to highest metabolic activity. This method turned out a valuable tool for assessment of metabolic activity of cells in anaerobic and microaerophilic conditions, enabling us to select strains with desired activity in microaerophilic and anaerobic processes or make combinations of both features. The values for the redox potential of investigated 10 *C. bifermentans* strains are presented in Figure 3. All strains demonstrated high redox potentials - both in anaerobic and microaerophilic conditions. It means that the cells are able to survive in the presence of oxygen. Additionally, in majority of the investigated strains the redox potential is similar for both types of cultivating, the presence of oxygen does not decrease the metabolic activity of cells. From the industrial point of view, it is an important property of *C. bifermentans* because work with an obligate anaerobe is difficult on a large scale, while this feature limits the possibilities of application of strictly anaerobic strains.

Morphological and physiological properties of isolated strains

All experiments were done in microanaerobic conditions. All of 10 obtained *C. bifermentans* strains cultivated on TSC medium formed opaque, black, circular, low convex colonies with entirely to slightly undulated margins. Spores are oval, central to subterminal. The biochemical features such as the motility of these strains, urease and indole production, as well as results of sugar assimilation and fermentation tests are presented in Table 3. As we can see, these strains have different features. Some of them are able to motile (*C. bifermentans* 371, 376, 546, and 549), while others are not. All investigated isolates were not able to produce indole and they were urease-negative. One strain, *C. bifermentans* 540,

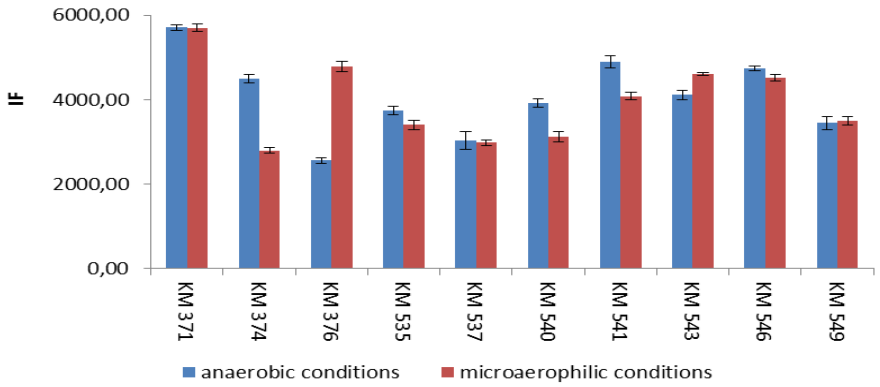

Figure 3. The redox potential of *C. bifermentans* strains cultivated both in microaerophilic and anaerobic conditions (IF - fluorescence intensivity).

Table 3. Characterization of isolated *C. bifermentans* strains.

Strain number/properties	371	374	376	535	537	540	541	543	546	549
Motility	+	-	+	-	-	-	-	-	+	+
Urease production	-	-	-	-	-	-	-	-	-	-
β-Glucosidaze hydrolysis	+	+	+	+	+	+	+	+	+	+
Gelatin hydrolysis	+	+	+	+	+	+	+	+	+	+
Glucose assimilation	+	+	+	+	+	+	+	+	+	+
Arabinose assimilation	+	+	+	+	+	-	+	+	+	+
Mannose assimilation	+	+	+	+	+	-	+	+	+	+
Mannitol assimilation	+	+	+	+	+	-	+	+	+	+
N-Acetylo-glukosamine assimilation	-	-	-	+	+	+	+	-	+	+
Maltose assimilation	+	+	+	+	+	+	+	+	+	+
Indole production	+	+	+	+	+	+	+	+	+	+
Glucose fermentation	+	+	+	+	+	+	+	+	+	+
Maltose fermentation	+	+	+	+	+	+	+	+	+	+
Fructose fermentation	+	+	+	+	+	+	+	+	+	+
Sorbitol fermentation	+	+	+	+	+	+	+	+	+	+
Arabinose fermentation	+	+	+	+	+	+	+	+	+	+
Mannose fermentation	+	+	+	+	+	+	+	+	+	+
Mannitol fermentation	+	+	+	+	+	+	+	+	+	+
Raffinose fermentation	+	+	+	+	+	+	-	+	+	+
Xylose fermentation	+	+	+	+	+	+	+	+	+	+
Inuline fermentation	-	-	-	-	-	-	-	-	-	-
Starch fermentation	-	-	-	-	-	-	-	-	-	-
Trehalose fermentation	-	-	-	-	-	-	-	-	-	-
Rhamnose fermentation	-	-	-	-	-	-	-	-	-	-

showed a number of different features in comparison with others, such as inability to assimilate arabinose, mannose, and mannitol while others are able to assimilate these sugars. In our work we also checked the ability of sugar fermentation in all 10 strains (glucose, fructose, maltose, sorbitol, arabinose, mannose, mannitol, raffinose, and xylose). Table 3 shows also the results from these experiments. However, when another carbon source, different than glycerol, was used, there was no 1,3-PD production.

Antibiotic sensitivity test

Antibiotic sensitivity testing aims to determine the susceptibility of an isolate to a range of potential therapeu-

Table 4. The results of antibiotics susceptible test.

Antibiotic/strain number	371	374	376	535	537	540	541	543	546	549
Erythromycin	+	+	+	+	+	+	+	+	+	+
Penicillin	+	+	+	+	+	+	+	+	+	+
Gentamycin	-	+	-	-	+	-	-	-	+	+
Ortho-tetracycline	+	+	+	+	+	-	-	-	+	+
Streptomycin	-	-	-	-	-	-	-	-	-	-
Ampicillin	+	+	+	+	+	+	+	+	+	+
Tetracycline	+	+	+	+	+	+	+	+	+	+
Chloramphenicol	+	+	+	+	+	+	+	+	+	+

+ Strain susceptible to antibiotic. - Strain resistant to antibiotic

tic agents. Because in the available literature there is many incoherent data about the sensitivity of *C. bifermentans* to some antibiotics, in this work the antibiotic sensitivity test was done for all 10 new isolated *C. bifermentans* strains.

The results of antibiotics susceptible test are presented in Table 4. All 10 strains are susceptible to erythromycin, penicillin, ampicillin, tetracycline, and chloramphenicol. Our results show that all 10 strains are resistant to streptomycin, three (*Clostridium bifermentans* 537, 546, and 549) are resistant to gentamycin, and only three (*Clostridium bifermentans* 540, 541, and 543) are resistant to ortho-tetracycline.

DISCUSSION

In the literature there is a lot of information about bacteria strains that are able to produce 1,3-PD (Biebl et al., 1999; Colin and Bories, 2000; Hao et al, 2008; Saxena et al., 2009; da Silva et al., 2009). The production of 1,3-PD from glycerol is generally performed under anaerobic conditions using glycerol as the sole carbon source in the absence of other exogenous reducing equivalent acceptors (34). A number of microorganisms can grow anaerobically on glycerol as the sole carbon and energy source. This group includes: *C. butyricum* (Colin and Bories, 2000), *C. pasteurianum* (Biebl et al., 1992), *Clostridium diols*, *Clostridium butylicum*, *Clostridium perfingens* (Hao et al., 2008), *Enterobacter agglomerans* (Barbirato et al., 1998), *Enterobacter aerogenes* (da Silva et al., 2009), *Klebsiella pneumonia* (Biebl et al., 1998), *Klebsiella oxytoca* (Homann et al., 1990), *Klebsiella aerogenes*, *Citrobacter freundii* (Malinowski, 1999), *Lactobacillus reuterii*, *Lactobacillus buchnerii*, *Lactobacillus collinoides*, *Pelobacter carbinolicus*, *Rautella planticola* (Saxena et al., 2009), and *Bacillus welchii* (da Silva et al., 2009). However, such strains as *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Klebsiella aerogenes*, *Enterobacter agglomerans*, *E. aerogenes*, *Citrobacter freundii*, *Lactobacillus reuteri*, *Lactobacillus buchnerii*, *L. collinoides*, *Pelobacter carbinolicus*, and *Rautella planticola* can produce 1,3-PD in microaerophilic fermentation (Biebl et al., 1998; da Silva, 2009; Saxena et al., 2009). Unfortunately, a key problem here is that the best 1,3-PD producers are pathogenic.

In our work we examined the natural environment in search for strains which are able to produce 1,3-PD. On the whole the natural environment is a good source of industrially useful strains. Additionally, there is a huge probability that even anaerobic strains isolated from this source have a natural tolerance to small amounts of oxygen. Thus we selected 10 strains of *C. bifermentans* from silage and forest soil. In the literature there are only a few papers describing the isolation of *C. bifermentans* from the natural environment, namely from California desert tortoise (Dezfulian et al., 1994; Chamkha et al., 2001) but there is no data about the ability of *C. bifermentans* to produce 1,3-PD. *C. bifermentans* are described as strictly anaerobic species (Lewis et al., 1996; Chang et al., 2000; Zhao et al., 2003). During our experiments it occurred that these strains are able to grow and maintain their metabolic activity in the presence of a small amount of oxygen (5%). From the industrially useful point of view, such a feature is very profitable because maintenance of strictly anaerobic conditions on a large scale is difficult and expensive. The metabolism and physiology of *C. bifermentans* species is not quite known (for example, there is no unequivocal information about the glycerol pathway in *C. bifermentans* cells). Thus, we decided to investigate this phenomenon. During a glycerol fermentation test we found out that our isolates produced more than 9 g/L of 1,3-PD without any optimization processes. Similar results are presented in the preliminary studies by Mu et al. (2006) on *K. pneumoniae* DSM 2026 isolated from a garden pond (9.4 g/L of 1,3-PD). In the glycerol fermentation by *C. bifermentans* 1,3-PD is a main metabolite, it is produced in reductive branch of glycerol metabolism pathway. Nevertheless, our isolates are also able to form other products, such as organic acids (acetic, lactic, formic, and succinic acid) and alcohol (ethanol) (Leja et al., 2011). These by-product

products are synthetized in the oxidative branch of glycerol metabolic pathway (Zeng and Biebl, 2002). The *Clostridium* strains growing on glycerol typically produce a variety of metabolic end-products, such as n-butanol, 1,3-PD, ethanol, acetic acid, butyric acid, and succinic acid (Biebl et al., 1999; Biebl, 2001; Biebl et al., 2002; Liu et al., 2007; Zhang et al., 2007; Kubiak et al., 2012). All these by-products are associated with a loss in 1,3-PD relative to acetic acid, in particular ethanol and butanol, which do not contribute to the $NADH_2$ pool at all (Zeng and Biebl, 2002). It is an example of a redox-balanced process in this pathway. Although the pathways for succinate and ethanol are equivalent regarding the overall redox balance, the energetic contribution of the ethanologenic pathway is much higher, as 1 ATP is produced per each molecule of glycerol converted into ethanol, while the production of energy in the succinate pathway is limited to the potential generation of a proton motive force by fumarate reductase (da Silva et al., 2009). An important by-product in this fermentation is acetic acid. The yield of 1,3-PD depends on the combination and stoichiometry of the reductive and oxidative pathways. It is proved that the combination of 1,3-PD generation with acetic acid as the sole by-product of the oxidative pathway results in the maximum yield of 1,3-PD (Shrestha and Sharma, 1995). Thus, the acetic acid is necessary for 1,3-PD production. The results of our experiments confirmed this fact: all 10 investigated strains are able to produce 1,3-PD and also synthesiz acetic acid. Concluding: the production of metabolites is necessary for bacteria strains. Especially important is the conversion of glycerol to 1,3-PD because it provides energy for cell growth (Biebl et al., 1999). The production of organic acids by microorganisms plays a key role in the process of controlling of appearance of other genus of bacteria in particular environments (Prevot and Malgras, 1950). Siragusa and Dickson (1992) stated that a small amount of short-chain organic acids resulted in the reduced growth of coexisting non sporogenes microflora.

Moreover, physiological properties of *C. bifermentans* are not well investigated. In the literature there is incoherent data about such properties of *C. bifermentans* as the motility of these species, the ability of indole and urease production, the fermentation of saccharides and resistance to antibiotics. Regan and Crawford (1994) found out that *C. bifermentans* strains are able to motile. The same kind of data is presented in the work of Prevot and Malgras (1950). However, Brooks and Epps (1958) inform us that some strains of *C. bifermentans* are motile while some are non-motile. The results of our work thus confirm the observations of Brooks and Epps (1858). Among our ten strains, four were motile and six were not. Regan and Crawford (1994) and Nachman et al. (1989) described C. *bifermentans* strains as indole positive. Brooks and Epps' (1958) results remain doubtful. They described this result as weakly positive. All our isolates were indole-positive and urease-negative, which is a finding corresponding to the data presented in papers by Brooks et al. (1969) and Brooks and Epps (1958). Brooks and Epps (1958) also investigated the ability of *C. bifermentans* to saccharide fermentation. They stated that their strains fermented glucose, fructose, maltose, glycerol, and sorbitol. All our strains were able to carry out these saccharide fermentations. The strains isolated by Chamkha et al. (2003) from oil mill wastewaters were also able to ferment glucose, fructose, mannose, maltose, sorbitol, and additionally myo-inositol and ribose. Nachman et al. (1989) and Regan and Crawford (1994) investigated also the sensitivity of *C. bifermentans* strains to antibiotics. The strains investigated by Nachman et al. (1989) were susceptible to erythromycin, penicillin, ampicillin, and tetracycline. The strains described in the work of Regan and Crawford (1994) were similarly susceptible to penicillin, ampicillin, and additionally to chloramphenicol. On the other hand, these strains were resistant to tetracycline. Other antibiotics have not been tested yet. Similar results were observed in our experiments: all ten isolates were susceptible to erythromycin, penicillin, ampicillin, chloramphenicol and also tetracycline. Probably, the incoherence in properties of *C. bifermentans* in different research results is a symptom of a great variety of isolated *C. bifermentans* strains. It may also be connected with different sources of their isolation.

Conclusions

The natural environment is a good source of industrially useful strains, such as *C. bifermentans*, which in our work was isolated from forest soil and sewage. *C. bifermentans* so far are not well known bacteria, and the predominant part of work on these species was carried out in the early 1950'. The results obtained by different scientists are not congruent. Thus, when it turned out that *C. bifermentans* is capable of 1,3-PD production (a feature which has not been described by any other scientists before), we decided to investigate physiological and biochemical properties of all the obtained isolates. Additionally, during this work it was found out that these bacteria are able to survive in the presence of oxygen, maintaining their ability to metabolites production (including 1,3-PD). Now, because of other interesting properties of these strains, such as effective production of lactic acid from mannitol, we are continuing our research on *C. bifermentans.*

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Multiple-locus variable-number tandem-repeat analysis of *Bacillus anthracis* isolated from a human-animal anthrax outbreak in the Luangwa valley of Zambia

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The incidence of anthrax, caused by *Bacillus anthracis*, in human and animal population of Zambia has increased recently. In this study, 34 strains of *Bacillus anthracis* from soil, hippopotamuses and humans, isolated in the 2011 outbreak were analyzed using the multiple-locus variable-number tandem repeat analysis. The analysis revealed that a single anthrax clone may have been involved in the epidemic. Considering the cyclical nature of *B. anthracis*, a link could be established with anthrax spores in soil getting ingested by hippopotamuses, followed by human contact resulting into an animal-human epidemic. These data confirm the importance of molecular typing methods for in-depth epidemiological analyses of anthrax epidemics.

Key words: *Bacillus anthracis,* hippopotamuses, variable number tandem repeat (VNTRs), epidemic, human.

INTRODUCTION

Bacillus anthracis is a zoonotic disease-causing agent for anthrax. It is a spore forming bacterium that causes disease in wild and domestic animals (Koehler, 2002; Habrun et al., 2011). Animals especially herbivores are mostly infected by oral ingestion of soil contaminated with anthrax spores, while humans are infected through contact with contaminated animal products (Spencer, 2003). In humans, the bacterium causes three major types of anthrax namely inhalational, gastrointestinal and cutaneous (Baykam et al., 2009).

B. anthracis has a worldwide dissemination as it is easy to propagate and has a history of being used as an agent of bioterrorism (Gierczynski et al., 2009). With this background, it is therefore important that the bacteria be identified and typed accurately in order to determine microbial forensics associated with disease and act appropriately in controlling the infection (Koehler, 2002). To enhance such an understanding, the phenotypic and

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genetic properties of isolates from one source to the other must be determined. *B. anthracis* is highly monomorphic in nature and differentiation of isolates from different regions has proved to be difficult (Brodzik and Francoeur, 2011). However attempts have been made to develop techniques that can resolve the minor genetic differences that can be found in *B. anthracis.* Genetic differentiation among very closely related individuals such as *B. anthracis* requires the use of molecular markers that exhibit very high diversity (Keim et al., 2004). These techniques include; multiple-locus variable-number tandem repeat analysis (MLVA), amplified fragment length polymorphism (AFLP), single nucleotide repeats (SNR) and single nucleotide polymorphism (SNP) (Vos et al., 1995; Okinaka et al., 2008). SNP detection is facilitated by whole genome discovery approaches which are useful for identifying long branches or key phylogenetic positions (Keim et al., 2004) while AFLP has good resolution analysis that require the use of MLVA. The MLVA is the most suitable technique to differentiate and subtype *B. anthracis* strains (Lindstedt, 2005). One powerful feature of the MLVA system is the ability to simultaneously employ multiple variable number tandem repeat (VNTRs) markers that exhibit varying levels of diversity and, therefore, high resolving power. In this study, we analyzed and compared isolates of *B. anthracis* from wildlife (hippopotamus), soil and humans reported in a human-animal anthrax outbreak in Zambia (Hang'ombe et al., 2012). Anthrax outbreaks have been recorded in Zambia, since 1989 (Turnbull et al., 1991; Turnbull, 1998; Tuchili et al., 1993; Siamudaala et al., 2006) without any report of direct molecular epidemiological linkage of *B. anthracis* strains from an epidemic scenario in Zambia.

MATERIALS AND METHODS

In this study, we analyzed 34 *B. anthracis* strains associated with a single anthrax epidemic in wildlife and human populations. A vaccine Sterne strain was used as a positive control and was compared with the epidemic isolates. Each *B. anthracis* strain was streaked onto 5% sheep blood agar plates and then incubated at 37°C for 24 h. Genotyping was done as previously described (Keim et al., 2004). The cultured isolates were inactivated by heating at 95°C for 30 min after which DNA was extracted using a DNA extraction kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. Following DNA extraction, a panel of 21 VNTR markers was used in this analysis (Table 1). These were variable repeat region (*vrr*) markers; *vrrA, vrrB1, vrrB2*, *vrrC1*, *vrrC2*, CG3, pXO1-*aat*, (Schupp et al., 2000) and Ceb-Bams (CB); CB-1, CB-3, CB-5, CB-7, CB-13, CB-15, CB-21, CB-22, CB-23, CB-24, CB-25, CB-28, CB-30 and CB-31 (Le Fleche et al., 2001). The PCR amplification was performed using the Phusion^{TM} flash high fidelity PCR master mix (Finnzymes Oy, Finland). The reactions were performed in a final volume of 10 µl containing 5 µl Phusion flash PCR master mix, 0.5 µM of primer sets in 1 µl volume of each and 2 µl of PCR water. The Piko^{TM} thermal cycler (Finnzymes Instruments Oy, Finland) was programmed at 95°C for 10 s for initial denaturation, followed by 35 cycles consisting of 95°C for 1 second, 58°C (changed according to the primers (Table 1)) for 5 s and 72°C for 15 s. Final extension was given 72°C for 1 minute. The thermal PCR-profile was used for all the VNTR markers. The amplicons were visualized on 1.5% agarose gel (MP Biomedicals, Eschwege, Germany), stained with ethidium bromide and evaluated under UV transilluminator. The estimation of the sizes of PCR products was done according to the migration pattern of a 100-bp DNA ladder.

RESULTS AND DISCUSSION

In this study, we used the MLVA genotyping comprising 21 VNTR markers to understand the relationship of 34 strains of *B. anthracis* isolated from soil (13 strains), hippopotamuses (16 strains) and human (5 strains) during an anthrax epidemic. Of these isolates, no difference was observed on a panel of 21 VNTR markers. The positive control isolate which is the vaccine strain (Sterne strain) had an observable difference with the outbreak isolates. The difference was observed with the CG3 marker where the Sterne vaccine was positive for CG3, while the outbreak strains were negative (Table 2). Further differences were noted with other markers which amplified a different molecular weight amplicon. The markers with different weight amplicons when compared with the Sterne vaccine strain were, CB-1, CB-3, CB-15, CB-21, CB-22, CB-23, CB-30 and CB-31. This MLVA analysis conducted on the isolates from human, soil and hippopotamus revealed relatedness using 21 VNTRs. The results indicated that all 34 isolates belonged to a single clone. The outbreak strains had the pXO1 and pXO2 plasmids required for virulence. Their presence indicates strain pathogenicity (Keim et al., 1997; Jackson et al., 1997; Habrun et al., 2011) as opposed to the vaccine strain which is negative for the pXO2 plasmid as expected. Analysis of other variable repeats found within *B. anthracis* provides a valuable tool to understand the epidemiology of anthrax outbreaks (Jackson et al., 1997). In this study, the confirmation through MLVA, indicates the outbreak may have been due to a single close. The results may also indicate that changes from one host to another as a result of genetic instability at the locus during the culture or infection process are rare. Such sequence stability suggests the utility of the VNTR regions as markers to determine the source of an infection or outbreak.

Conclusion

The cyclical nature of anthrax outbreaks determined by climatic factors such as extreme weather changes like drought followed by heavy rains, may lead to generation of high anthrax spore concentration causing disease in grazing animals such as hippopotamus which are bulky grazers. This outbreak indicated a single clonal source transmission, probably from soil to hippopotamuses and finally to humans through consumption of contaminated meat. The data presented here are limited and therefore call for continuous monitoring of human and animal clinical isolates and their environment (including soil) to

Table 1. Primers used in this study for MLVA analysis.

VNTR locus	Primer sequences (5' to 3')	Annealing temperature (°C)
vrrA	F: CACAACTACCACCGATGGCACA R: GCGCGTTTCGTTTGATTCATAC	60
vrrB1	F: ATAGGTGGTTTTCCGCAAGTTATTC R: GATGAGTTTGATAAGAATAGCCTGTG	61
vrrB2	F: CACAGGCTATTCTTTATCAAACTCATC R: CCCAAGGTGAAGATTGTTGTTGA	60
vrrC1	F: GAAGCAAGAAAGTGATGTAGTGGCA R: CATTTCCTCAAGTGCTACAGGTTC	62
vrrC2	F: CGAGAAGAAGTGGAACCTGTAGCAC R: GTCTTTCCATTAATCGCGCTCTATC	62
CG3	F: TGTCGTTTTATCTTCTCTCTCCAATAC R: AGTCATTGTTCTGTATAAAGGGCAT	59
pXO1	F: CAATTTATTAACGATCAGATTAAGTTCA R: TCTAGAATTAGTTGCTTCATAATGGC	57
CB-1	F: GTTGAGCATGAGAGGTACCTTGTCCTTTT R: AGTTCAAGCGCCAGAAGGTTATGAGTTATC	66
CB-3	F: GCAGCAACAGAAAACTTCTCTCCAATAACA R: TCCTCCCTGAGAACTGCTATCACCTTTAAC	64
CB-5	F: GCAGGAAGAACAAAAGAAACTAGAAGAGCA R: ATTATTAGCAGGGGCCTCTCCTGCATTACC	64
CB-7	F: GAATATTCGTGCCACCTAACAAAACAGAAA R: TGTCAGATCTCTAGTTGGCCCTACTTTTCCTC	63
CB-13	F: AATTGAGAAATTGCTGTACCAAACT R: CTAGTGCAATTTGACCCTAACTTGT	58
CB-15	F: GTATTTCCCCCAGATACAGTAATCC R: GTGTACATGTTGATTCATGCTGTTT	59
CB-21	F: TGTAGTGCCAGATTTGTCTTCTGTA R: CAAATTTTGAGATGGGAGTTTTACT	58
CB-22	F: ATCAAAAATTCTTGGCAGACTGA R: ACCGTTAATTCACGTTTAGCAGA	57
CB-23	F: CGGTCTGTCTCTATTATTCAGTGGT R: CCTGTTGCTCCTAGTGATTTCTTAC	62
CB-24	F: CTTCTACTTCCGTACTTGAAATTGG R: CGTCACGTACCATTTAATGTTGTTA	59
CB-25	F: CCGAATACGTAAGAAATAAATCCAC R: TGAAAGATCTTGAAAAACAAGCATT	56
CB-28	F: CTCTGTTGTAACAAAATTTCCGTCT R: TATTAAACCAGGAGTTACTTACAGC	59
CB-30	F: AGCTAATCACCTACAACACCTGGTA R: CAGAAAATATTGGACCTACCTTCC	61
CB-31	F: GCTGTATTTATCGAGCTTCAAAATCT R: GGAGTACTGTTTGTTGAATGTTGTTT	59

help draw sound conclusions on the exact spread of *B. anthracis*.

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Table 2. Genotyping results of *Bacillus anthracis* strains isolated from soil, hippopotamus and humans with the 21 VNTR locus.

Strain	VNTR markers																				
	vrrA1	vrrB1	vrrB2	vrrC1	vrrC2	CG3	pXO1	CB-1	CB-3	CB-5	CB-7	CB-13	CB-15	CB-21	CB-22	CB-23	CB-24	CB-25	CB-28	CB-30	CB-31
Outbreak isolates	+	+	+	+	+	_	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sterne Vaccine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

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Full Length Research Paper

First report of isolation and identification of *Brevundimonase* (*Pseudomonas*) *diminuta* from collected nasopharyngeal specimens in suspected patients to pertussis

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Brevundimonase diminuta **as an opportunistic environmental bacterium, due to the increase in isolation rate from clinical specimens and its antibiotic resistance is considered as a new threat to human health care. After performing conventional phenotypic methods and molecular approaches, antibiotics resistance pattern on the identified *Brevundimonase* isolates was performed as minimum bactericidal concentration by disk diffusion method. Out of 1084 nasopharyngeal specimens, 2/1084 (%0.18) *Brevundimonase diminuta,* and 1/1084 (%0.09) *Brevundimonase subvibrioides* were isolated. Evaluation of the resistance pattern from isolated *Brevundimonase* strains indicated that: levofloxacin, ciprofloxacin, ampicillin, amikacin (Excluding *B. subvibrioides*), chloroamphenicol and ceftazidime had poor susceptibility results. Also azythromycin, gentamycin, sulfamethoxazole/trimethoprim (Excluding *B. subvibrioides*), ciprofloxacin, kanamycin showed good susceptibility results against *Brevundimonase* isolated strains. In conclusion, Although, *Brevundimonase diminuta* rarely has been isolated from other clinical specimens, but, due to reports of resistance to some of antimicrobial agents in this genus all laboratories should be equipped for the identification and evaluation of susceptibility patterns of this species. It is first the report of isolated *Brevundimonase diminuta* from nasopharyngeal specimen from suspected patients to pertussis in Iran.**

Key words: *Brevundimonase diminuta*, nasopharyngeal specimens, *16srRNA* primer.

INTRODUCTION

According to the original description in 1984, *Brevundimonase spp* were classified as members of the genus *Pseudomonas* (Palleroni, 1984; Segers et al., 1994). *Bervundiomonase* (*Pseudomonas*) *diminuta* is a non-fermentative gram negative bacterium with 0.5 × 1-4 µm size. Moreover, due to the presence of one polar

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Table 1. Clinical characteristics of patients infected with *B. diminuta* strains.

Sample	Age	Sex	Source	Signs and Symptoms	Province	Utilized Antibiotics	Date
Sample 1	5 m	Male	Nasopharyngeal	Fever (39) cough vomiting	Khuzestan /abozar	Azythromycin- ampicillin	2010
Sample 2	3 year	Male	Nasopharyngeal	Fever (38.9) cough vomiting	Mazandaran/babol	Co-amoxiclave-erytromycin-cefixime	2009

M, month; Y, year

flagellum, it is classified as motile bacteria. *Brevundimonase diminuta* previously described as an opportunistic environmental bacterium and assigned in the genus of *Pseudomonas* (lefson and Hugh, 1954; Segers et al., 1994; Gilligan et al., 2003; Han and Andrade, 2005). Recently, due to the presence of unique *16srRNA* gene, *Brevundimonase spp* have been reclassified (Lee et al., 2011). Han and Andrade (2005) reported that, *B. diminuta* has been isolated and identified from water and clinical specimens. Pathogenecity of *B. diminuta* has not been determined yet and due to small size of the bacterium routinely is used as an indicator of the efficiency of water filters (Holt et al., 1994). Segers et al. (1994) reported that, *B. diminuta* strains could be isolated from human specimens such as human urine (Goteborg, Sweden), blood plasma (Slagelse, Denmark), human ear (Boras, Sweden), hemoculture (Hospital, Brussel, Belgium), pleura liquid (Hospital, Brussel, Belgium) and blood from patients with endocardities (Culture Collection of the University of Goteborg, Department of Clinical Bacteriology, University of Goteborg, Goteborg, Swede (CCUG 1797). *Brevundimonase vesicularis* from environmental (United States) and human specimens including pleura liquid (Hospital, Brussel, Belgium), Vaginal swab (Hospital, Brussels, Belgium), hemoculture (Hospital, Brussel, Belgium) and urinary bladder (Hirudu sp) has been isolated.

This study generally designed for identification and confirmation of the presence of *Brevundimonase diminuta* from nasopharyngeal samples in suspected patients to pertussis based on the conventional biochemical tests and molecular approaches and further analysis of antibiotic resistance which housed them.

MATERIALS AND METHODS

Routine procedure for isolation and identification of *Bordetella pertussis* in Pasteur Institute of Iran

Nasopharyngeal specimens from suspected patients were collected by sterile Dacron swabs. Subsequently, samples immediately emulsified in Riegan Lowe Medium (Difco) as transport medium. All samples were suspended in phosphate buffer solution (PBS). DNA extracting process directly performed (in nasopharyngeal samples from suspected patients to pertussis) by high pure DNA extraction kit (Roche, Diagnostics GmbH, Mannheim, Germany). Taq man real time PCR assay optimized for rapid detection of *B. pertussis* was used. For precise determination of *B. pertussis* in clinical samples, insertion sequence 481(IS 481) and sequence BP 283 (Codon gene between tyolase region) were targeted by using of specific primers and probes (Reischl et al., 2001, Probert et al., 2008). Positive strains for pertussis were affirmed by conventional phenotypic methods including grow in Bordet gengou agar with and without 40µg/ml cephalexin. As well, colony morphology, biochemical activity tests consisting of oxidase and catalase activity, gram stain, utilizing scheme for amino acids and carbohydrate fermentation and other substrate by API 20 E (BioMerieux, Inc., Hazelwood, MO) were performed. All isolated *B. pertussis* strains reconfirmed by serological test using antiserum kit (BioMerieux, Inc., Hazelwood, MO).

Identification of *Brevundimonase diminuta*

Conventional biochemical tests were done according to the Bergeys Manual of Determinative Bacteriology recipes and further isolates were confirmed using API 20 E technology (BioMerieux, Inc., Hazelwood, MO) (Holt et al., 1994). Clinical characteristics of patients and Biochemical characterization of *B. diminuta and B. subvibrioides* isolated strains are listed in Tables 1 and 2.

Antibiotics susceptibility test

Susceptibility testing for *B. diminuta* isolated strains was analyzed using disk diffusion method with ampicillin 10 µg, amikacin 10 µg, chloramphenicole 30 µg, trimethoprim/sulfamethoxazole 1.25 µg + 23.75 µg, ciprofloxacin 5 µg, levofloxacin 5 µg, gentamycin 120 µg, kanamycin 30 µg, imipenem 10µg, azythromycin 15 µg, cefepime 30 µg, ceftriaxone 30 µg, ceftazidime 30 µg and cefotaxime 30 µg disks (BD BBL^{TM} Sensi Disc^{TM}). Antimicrobial susceptibility using disk diffusion method was performed according to the CLSI standard guide line (CLSI, 2010). *Pseudomonase aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were included as control strains in each run. Evaluation of results was also performed considering that proposed by the manufacture recommendation break point set for *P. aeruginosa*.

Molecular techniques

Genomic DNA was extracted from each isolates using a comer-cial kit. Briefly, pure identified colonies were emulsified in 500µl PBS. Cells harvested by centrifugation at 8000 × *g* for 5 min. Subsequently, pellets consisting bacterial cells were used for DNA extraction process. Total DNA extraction was completed using a high pure DNA extraction kit (Roche, Diagnostics GmbH Mannheim, Germany). Purified DNA was dissolved in 50µl distilled water.

Table 2. Biochemical characterization of *B. diminuta and B. subvibrioides* isolated strains from patients.

Test	Substrate	*B. diminuta*		*B. subviridis*
		Sample 1	Sample 2	Sample 3
Biochemical tests (Tube tests)	MAC agar	+	+	+
	Citrate	-	-	+
	Motility	+	+	+
	Indol	-	-	-
	Oxidase	+	+	+
	Catalase	+	+	+
	TSI	Alk/Alk	Alk/Alk	Alk/Alk
API 20 E test	ONPG	-	-	-
	ADH	-	-	-
	LDC	-	-	-
	ODC	-	-	-
	CIT	-	-	+
	H_2S	-	-	-
	URE	-	-	+
	TDA	-	-	-
	IND	-	-	-
	VP	-	-	-
	GEL	-	-	-
	GLU	-	-	-
	MAN	-	-	-
	INO	-	-	-
	SOR	-	-	-
	RAH	-	-	-
	SAC	-	-	-
	MEL	-	-	-
	AMY	-	-	-
	ARA	-	-	-

ONPG, ortho-Nitrophenyl-β-galactoside**; ADH ,**amino acid decarboxylations**; LDC,** lysin decarboxilase**; ODC ,**ornithin decarboxilase**; CIT,** citrate**; H_2S,** Hydrogen sulfide**; URE,** urea**; TDA,** tryptophan deaminase**; IND ,**indole**; VP,** voges proscauer**; GEL,** gelatin hydrolysis**; GLU,** glucose**; MAN,** mannitol**; INO,** inositol**; SOR,** sorbitol**; RAH,** rhamnose**; SAC,**sucrose**; MEL,** melibiose**; AMY,** amygdalin**; ARA,** arabinose**; ALK,** alkalin;

Quantity of extracted DNA was measured by NANO-DROP ®ND-1000 instrument (spectrophotometer 1000, USA) and adjusted to 500 ng/µL^{-1}.

Amplification of *rrs* gene and sequencing process

Purified DNA was evaluated by polymerase chain reaction (PCR) utilizing universal *rrs* gene (*16srRNA*). Sequences of forward and reverse primer for 1475 bp PCR product were 5′-AGAGTTTGATCCTGGCTCAG-3′ and 5′-GGTTACCTTGTTACGACTT-3′ (Tsai et al., 2004). Amplification protocol originally described by previously published study (Tsai et al., 2004). PCR was performed using 2 µl genomic DNA as template in total volume 25 µl containing; 12 µl master amplicon (Biolab, New England, UK), Primer Forward 1µm, Primer Reverse 1 µm and 9 µl mineral oil. PCR process was optimized with purified DNA of *E.coli* ATCC 25922 as positive genotype. Amplification was performed on Gene Amp PCR system (Applied Biosystem, USA) using a program as follow: an initial cycle of denaturation 95°C for 5 min followed by 35 cycles of 95°C for 1 min, 50°C for 1 min and 72°C 1 min, with final extension step of 72°C for 10 min. Amplified products (1475 bp) were visualized on 1.5% agarose gel (Max pure agarose, Spain) stained by etidium bromide using Gel logic 212pro. Figure 1 showed amplified products of *rrs* gene.

Sequencing

PCR amplification from DNA preparations of *rrs* yielded corresponding products 1475 bp (Figure 2) were sequenced following the purification of PCR products using of purifying kit (QIAquick PCR Purification Kit, Qiagen). Sequencing process was accomplished by the dye terminal method in an ABI 377 sequencer and sequencing results interpreted through a query to the Gen Bank local alignment search tool (BLAST) (blast.ncbi.nlm.nih.gov/Blast.cgi?).

RESULTS

Out of 1084 nasopharyngeal collected samples using conventional biochemical and molecular tests, 12 bacteria

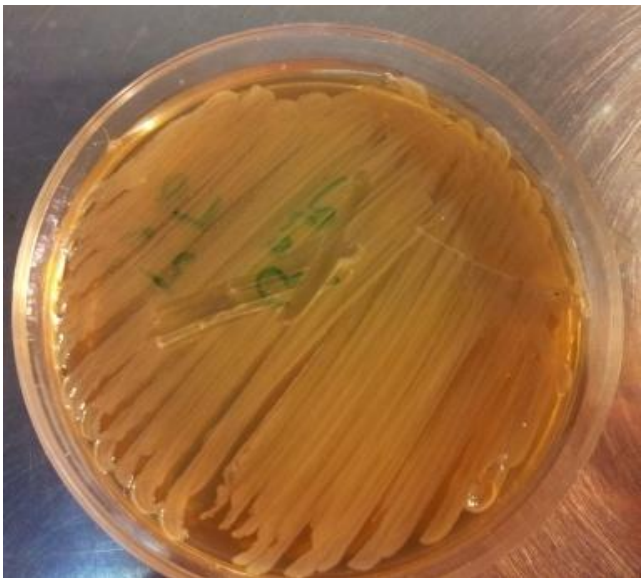

Figure 1. *Brevundimonase diminuta* grown colonies on Mueller Hinton medium.

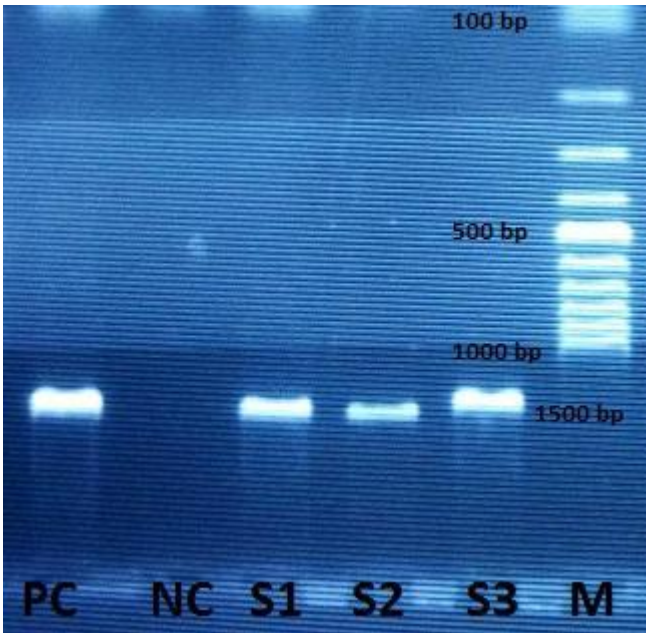

Figure 2. Generated *rrs* gene from suspected *Brevundimonase spp,* isolates. M, marker 100bp; NC, negative control (distilled water); PC, positive control (*E.coli* ATCC 25922); S1, 2 *B. diminuta*; S3, *B. subvibrioides*.

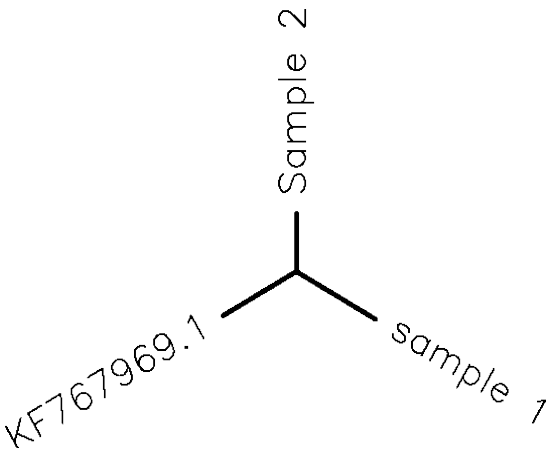

Figure 3. Unrooted phylogenic tree provided by Biology Workbench software. *KF767969*.1 served as *16srRNA* sequence of B. *diminuta.*

isolates included: 2/1084 (0% 18) *Brevundimonase diminuta* and 1/1084 (0% 09) *Brevundimonase subvibrioides,* 8/1084 (%0.73) *Bordetella pertussis*, 1/1084 (0.09%) *Bordetella parapertussis* were detected.

After PCR process and sequencing of generated amplicon, using online software and comparison with a data base identification of isolated *B. diminuta* and *B. subvibrioides* strains with match point ≥95, were confirmed. Subsequent comparison with the results obtained by biochemical and molecular methods two *Brevundimonase diminuta* isolates, the similar results were obtained which indicates that the results of both biochemical and molecular tests were correct. Unrooted phylogenic tree are shown in Figure 3. After performed disk diffusion method, azythromycin, gentamicin and sulfamethoxazole/trimethoprim have shown an appropriate inhibitory effect against *B. diminuta* isolated strains. On the contrary, antibacterial agents such as levofloxacin, ciprofloxacin, ceftazidime, ampicillin and amikacin due to low activity against *B. diminuta* strains have not detected for suitable drug treatment process.

Susceptibility pattern of the mentioned antibiotics against *B. subvibrioides* strain indicated that chloroamphenicol, azythromycin, ampicillin, sulfamethoxazole/trimethoprim, levofloxacin, ceftazidime and ciprofloxacin were not sufficient inhibitory effect against isolated strain. Also *B. subvibrioides* isolated strain has good susceptibility results against some of antibiotics such as gentamycin, kanamycin, cefepime and ceftriaxone. Detailed data are listed in Table 3.

DISCUSSION

Since 1994, according to the presence of unique *16srRNA* gene *Brevundimonase* spp have been reclassified (Han and Andrade, 2005). *Brevundimonase diminuta* and *B. versicularis* as environmental opportunistic bacteria commonly were isolated from immunocompromised patients (Holt et al., 1994; Han and Andrade, 2005).

Previously, *B. diminuta* was isolated from human specimen such as sputum in cystic fibrosis patients, urine, pleura effusion and blood (Chi et al., 2004; Han and Andrade, 2005; Menuet et al., 2008). According to the previously published study by Shayegani (1978), API

Table 3. Evaluation of antibiotic resistance pattern on *B. diminuta* and *B. subvibrioides* isolated strains.

Test	Antibiotics/ disc potency	*B. diminuta*		*B. subvibrioides*
		Sample 1 interpretation /Zone diameter	**Sample 2** interpretation /Zone diameter	**Sample 3** interpretation /Zone diameter
Antibiotic susceptibility pattern (disk diffusion)	IMI /10 µg	S(≥16)	S(≥16)	I(14-15)
	C /30 µg	I(13-17)	R(≤12)	R(≤12)
	AZH / 15 µg	S(≥18)	S(≥18)	R(0)
	AP/10 µg	R(0)	R(0)	R(0)
	CPM /30 µg	S(≥18)	S(≥18)	S(≥18)
	GM /120 µg	S(≥1)	S(≥1)	S(≥1)
	K /30 µg	S(≥18)	S(≥18)	S(≥18)
	TS /1.25 µg +23.75 µg	S(≥16)	S(≥16)	R(≤1)
	LVX / 5 µg	R(0)	R(0)	R(0)
	CRO /30 µg	I(14-20)	I(14-20)	I(14-20)
	Ak /10 µg	R(0)	R(0)	I(14-17)
	CIP / 5 µg	R(0)	R(0)	R(0)
	CAZ /30 µg	R(0)	R(0)	R(0)
	CTX /30 µg	I(15-22)	S(≥23)	S(≥23)

IMI, imipenem**; C,** chloramphenicol**; AZH,** azythromycin**; CPM,** cefepime**; GM,** gentamicin**; K ,**kanamycin**;TS,** trimethoprim/sulfamethoxazole**; LVX,** Levofloxacin**; AP,** ampicillin**; CIP,** ciprofloxacin**; CAZ,** ceftazidime**; CTX ,**ceftriaxime**; CRO,** ceftriaxone**; S,** sensitive**; I,** intermediate**; R,** resistant.

20 E technology was evaluated and presented as an efficient method for the identification of non-fermentative gram negative bacteria (Shayegani et al., 1978). In this study, using API 20 E technology two strains of *B. diminuta* and one strain of *B. subvibrioides* were identified according to the data included in the API 20 E data base. Although pathogenecity relationship of *B. diminuta* to pertussis not confirmed, and this bacterium frequently be isolated from mouth cavity but observation of mentioned bacterium from nasopharyngeal specimen in suspected patients to pertussis is considerable (Paster et al., 2002; Davis et al., 1997).

Recently, molecular techniques such as PCR and DNA sequencing have been utilized for fast and accurate detection of a wide range of microorganisms (Han and Andrade, 2005; Lee et al., 2011). In this study, generated amplicon by universal *rrs* primers was sequenced using sequencer instrument (ABI 377 sequencer) and following the sequence analysis through a query to the GenBank Basic Local Alignment Search Tool™ (BLAST), based on the www.NCBI.org, our phenotypic methods were affirmed and isolation and identification of *B. diminuta* were justifiable.

Because of multiple antimicrobial resistance characteristics in many isolates of *Brevundimonase* spp, selection of efficient antibiotics for treatment of related infections caused by these bacteria is difficult (Han and Andrade, 2005). Although, *Brevundimonase* species previously classified in the genus *Pseudomonas*, but routine antibacterial agents for the treatment of *Pseudomonas* infections (fluoroquinolones and ampicillin) have no detrimental effects on the this genera of bacteria (Davis et al., 1997; Lee et al., 2011).

Some published studies have shown that; *B. diminuta* has variable resistance against some antimicrobial agents such as; azteronam, ciprofloxacin, ceftazidime (Gilad et al., 2000, Chi et al., 2004; Han and Andrade, 2005; Choi et al., 2006; Almuzara et al., 2012). Susceptibility to the cefepime as 4th generation of cephalosporins was reported in one case (Choi et al., 2006).

Although mutations in GyrB and ParC subunit, have related to quinolones resistance, but in *P.aeruginosa* efflux pumps have been shown to be important for the quinolones resistance (Han and Andrade, 2005). In current study, azythromycin (exception one *B. subvibrioides*), gentamycin exhibited high affect in antibiogram process and could be considered as a treatment option. Detection of responsible genes or other mechanisms in our *B. diminuta* isolates need to more study (Almuzara et al., 2012).

Almunza (2012) reported that, only three *B. diminuta* environmental isolates were carrying metallo-β-lactamase genes (Almuzara et al., 2012).

In the present study, levofloxacin, ciprofloxacin, ampicillin, amikacin, chloroamphenicol and ceftazidime due to poor results at the antibiogram were described as an inappropriate treatment for related infections caused by Brevundimonase strains. Moreover, azythromycin (excluding *B. subvibrioides*), sulfamethoxazole/trimethoprim (excluding *B. subvibrioides*) and gentamicin, regarding good results in antibiogram process, were introduced as suitable candidates for the treatment of associated infec-

tions with isolated strains.

Conclusion

Due to the isolation rate of *B. diminuta* as the infectious agent, and attribution of human infections to this bacterium, bacterial identification and detection of antimicrobial susceptibility testing should be considered in the laboratories. Although most species in this genus are less pathogenic, but isolated strains from clinical specimens can be the alarm for the health system.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Mathematical modeling of some medium constituents and its impact on the production of vitamin B_{12} and folic acid by *Klebsiella pneumoniae* under solid state fermentation

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The present investigation involved testing the effect of different carbon sources (glucose, sucrose and starch) as well as different nitrogen sources (peptone, yeast extract and urea) on the production of vitamin B_{12} using *Klebsiella pneumonia* grown on mixture of agriculture wastes (wheat straw and rice bran). The results show that glucose was the best carbon source compared to the other di- and polysaccharides used. The best vitamin B_{12} output (63.87 µg/L) was obtained at 3 g/L glucose. On the other hand, peptone appeared to be the most suitable nitrogen, since it gave (57.8 µg/L) of B_{12} compared to the other nitrogen sources used. Statistical analysis for the obtained results had been carried out and the data showed that glucose and peptone additions were highly significant compared to the other sources under investigation.

Key words: Vitamin B_{12} production, solid state fermentation (SDF), bacteria.

INTRODUCTION

Vitamin B_{12} like other B vitamins is important for the metabolism. It helps in the formation of red blood cells and in the maintenance of the central nervous system (Wickramasinghe, 1995). The deficiency of vitamin B_{12} is associated with hematologic, neurologic and psychiatric manifestations. It is also a common cause of megaloblastic anemia (Atta et al., 2008). The deficiency may exert indirect cardiovascular effects in addition to the hematologic and neuropychatric manifestations (Nygard et al., 1997; Kałużna-Czaplińska et al., 2011).

On the other hand, folic acid is necessary for fertility in both men and women. In men it contributes to spermatogenesis but in women enhance oocycte maturation placentation (Ebisch et al., 2007). The biosynthesis of this essential nutrient is intricate, involved and, remarkably, confined to certain members of the prokaryotic world, seemingly never have to have made the eukaryotic transition (Martens et al., 2002). Bacteria are known for its de novo synthesis of vitamin B_{12} and folic acid through biological and biotechnological pathways. Masuda et al.

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Table 1. Effect of different glucose concentrations on production of vitamin B_{12} and Folic acid by *Klebsiella pneumoniae.*

Glucose concentration (g/L)	Vitamin B_{12} content (µg/L)	Folic acid content (µg/L)	Substrate consumption (%)
Control	38.74 ± 0.3	45.21 ± 0.2	11 ± 0.0
1.00	45.37 ± 0.7	50.91 ± 1.1	11 ± 1.0
2.00	56.03 ± 0.3	54.43 ± 0.8	12 ± 0.5
3.00	63.87 ± 0.9	58.96 ± 0.5	13 ± 1.0
4.00	60.28 ± 0.6	63.88 ± 0.2	13 ± 0.5
5.00	55.84 ± 2.3	61.72 ± 1.0	12 ± 0.0
P value	***	***	**
F test	226	281	5.8
LSD at 0.05	1.94	1.30	1.15

Initial pH 8. Incubation time 3 days, temp. 30ºC, substrate W.S+R.B (ratio 1:2 W/W) substrate weight 200 g/L.*** Highly significant p<0.001,** Moderate significant *p*<0.*01*, Control without addition.

(2012) investigated the extracellular production of folate, vitamin B12, and thiamine in cultures of lactic acid bacteria (LAB) isolated from nukazuke, a traditional Japanese pickle. They studied the relationships between the vitamin production and properties of LAB as tolerance to salts, ethanol, etc. Among 180 isolates of LAB, two strains were capable to produce 100 µg/L vitamins.

For the above mentioned factors, this work aims at the production of B_{12} and folic acid using some raw materials involving different agriculture wastes for the magnification of the production process.

MATERIALS AND METHODS

Microorganisms

The microorganism used in the current work *Klebsiella pneumoniae* was isolated from fecal specimen and identified in a previously discussed work (El-Sheekh et al., 2013). The fungus *Rhizopus nigricans* was provided from Natural and Microbial Products Department, National Research Center, (NRC) Dokki, Cairo.

Substrate preparation

The agriculture wastes (wheat straw and rice bran) were used in a mixture of 1:2 (w/w) respectively. These wastes were pretreated by cutting, grinding and alkaline hydrolysis with sodium hydroxide to separate the lignin components (Wanapate et al., 2009; El-Sheekh et al., 2013).

Cultivation

Erlenmeyer flasks (250 ml) each contain 20 g of the solid substrate and 100 ml sterile distilled water, which were previously inoculated with 1×10^6 spores of *Rhizopus*/ml and 4.5×10^8 bacterial cell/ml, were incubated statistically at 30°C for three days and the initial pH was adjusted at 8 (Keuth and Bisping, 1994; Atta et al., 2008; El-Sheekh et al., 2013). The flasks contents filtered and the filtrate was centrifuged at 4000 rpm for 10 min to separate the substrate from the culture medium. The substrate consumed was also determined after each experiment by calculating the percentage of the difference of weight before and after the experiment.

Estimation of vitamin B_{12} and folic acid

Radio-immunoassay (RIA) was used for the simultaneous quantitative determination of vitamin B_{12} and folic acid in comparison with standard according to the method described by Akatsuka and Atsuya (1989) and Wongyai (2000).

Statistical analysis

The obtained results were statistically analyzed using the analysis of variance (ANOVA) to determine the degree of significance for the variation of both vitamin B_{12} and folic acid yields. F test and the least significance different (LSD) at 0.05 level was also calculated for the mean values.All the statistical methods were applied according to the method described by Bishop (1983).

RESULTS AND DISCUSSION

Suitability of different carbon sources

Glucose concentration

Different glucose concentrations (1, 2, 3, 4 and 5 g/L) were tested in the production process of vitamin B_{12} by *Klebsiella pneumoniae*. It was clearly noticed in Table 1, that the addition of glucose enhanced the production of vitamin B_{12} and folic acid. The highest yield of vitamin B_{12} (63.87 µg/L) was obtained at 3 g/L. Further increase in glucose concentration was accompanied by reduction in the yield. The results of the statistical analysis of glucose addition were highly significant at p<0.001 for vitamin B_{12} and folic acid.

Sucrose concentration

The addition of different sucrose concentrations (1, 2, 3,

Table 2. Effect of different sucrose concentrations on production of vitamin B_{12} and Folic acid by *Klebsiella pneumoniae.*

Sucrose concentration (g/L)	Vitamin B_{12} content (µg/L)	Folic acid content (µg/L)	Substrate consumption (%)
Control	38.74 ± 0.3	45.21 ± 0.2	11 ± 0.0
1.00	38.74 ± 0.2	46.72 ± 0.8	11 ± 0.5
2.00	43.03 ± 1.0	49.39 ± 0.4	11 ± 1.0
3.00	47.12 ± 0.4	50.90 ± 0.5	11 ± 0.5
4.00	48.27± 0.3	51.77 ± o.4	12 ± 1.5
5.00	46.93 ± 0.9	51.08 ± 0.2	12 ± 1.0
P value	***	***	NS
F test	102	98	1
LSD at 0.05	1.07	0.82	1.58

Initial pH 8. Incubation time 3 days, temp. 30 °C, substrate W.S+R.B (ratio 1:2 W/W) substrate weight 200 g/L. ***Highly significant p<0.001,**Moderate significant p<0.01, Control without addition.

Table 3. Effect of different starch concentrations on production of vitamin B_{12} and Folic acid by *Klebsiella pneumoniae.*

Starch concentration (g/L)	Vitamin B_{12} content (µg/L)	Folic content (µg/L)	Substrate consumption (%)
Control	38.74 ± 0.3	45.21 ± 0.2	11 ± 0.0
1.00	40.88 ± 0.9	50.27 ± 0.3	11 ± 1.0
2.00	42.27 ± 0.6	50.79 ± 0.2	11 ± 1.5
3.00	43.93 ± 0.4	51.61 ± 0.5	12 ± 1.0
4.00	43.47 ± 0.3	51.13 ± 0.2	12 ± 0.5
5.00	42.89 ± 0.8	49.07 ± 1.0	12 ± 0.0
P value	***	***	NS
F test	31	68	1.2
LSD at 0.05	1.06	0.88	1.5

Initial pH 8. Incubation time 3 days, temp. 30°C, substrate W.S+R.B (ratio 1:2 W/W) substrate weight 200 g/L. *** Highly significant *p<0.001*, NSnon significant (p>*0.05*), control without addition.

4 and 5 g/L) were tested for vitamin B_{12} and folic acid production by *Klebsiella pneumoniae*. The results presented in Table 2 showed that the best Vitamin B_{12} yield (48.27 µg/L) was obtained at sucrose concentration 4 g/L. On the other hand, the folic acid yield was 51.77 µg/L at the same concentration.Further increase in the sucrose concentration was accompanied by slightdecrease in the outputs .The statistical analysis of sucrose addition showed that it was highly significant (p<0.001) for vitamin B_{12} and folic acid (Keuth and Bisping, 1993; Herranen et al., 2010).

Starch addition

The addition of different starch concentrations (1, 2, 3, 4 and 5 g/L) were investigated. The data in Table 3 revealed that the addition of this polysaccharide has low effect on the stimulation of the vitamin B_{12} and folic acid production. The maximum yields of vitamin B_{12} and folic acid were obtained at 3 g/L (43.93 and 51.61 µg/L), respectively. The statistical analysis of the sucrose addition results showed that it is highly significant (p<0.001) for B_{12} and folic acid, but non-significant at p>0.05 for the substrate used (Keuth and Bisping, 1994; Nygard et al., 1997; El-Sheekh et al., 2013).

Suitability of different nitrogen sources

Addition of different nitrogen sources (yeast extract, peptone and urea) were tested for their effects on the production of vitamin B_{12} and folic acid.

Yeast extract

In this experiment different concentrations of yeast extract (1, 2, 3, 4 and 5 g/L) were used to test the optimum production of vitamin B_{12} and folic acid. The results presented in Table 4 showed that the addition of 4 g/L enhanced the production of vitamin B_{12} and folic acid (46.76 and 55.51 µg/L) respectively, and accompanied by 13% substrate consumption (Keuth and Bisping, 1994; Nygard et al., 1997). The statistical analysis results revealed that the addition of yeast extract appeared to be highly significant (p>0.001) for both vitamin B_{12} and folic

Table 4. Effect of different yeast extract concentrations on production of vitamin B_{12} and Folic acid by *Klebsiella pneumoniae.*

Yeast extract concentration (g/L)	Vitamin B_{12} content (µg/L)	Folicacid content (µg/L)	Substrate consumption (%)
Control	38.74 ± 0.3	45.21 ± 0.2	11 ± 0.0
1.00	38.96 ± 1.1	52.74 ± 0.8	11 ± 1.0
2.00	43.00 ± 0.6	54.59 ± 0.4	12 ± 0.5
3.00	45.95 ± 0.8	54.63 ± 0.3	12 ± 1.0
4.00	46.67 ± 0.2	55.51 ± 0.6	13 ± 1.0
5.00	45.13 ± 0.5	51.69 ± 1.6	12 ± 1.5
P value	***	***	NS
F test	85	67	1.9
LSD at 0.05	1.17	1.43	1.70

Initial pH 8. Incubation time 3 days, temp. 30 °C, substrate W.S+R.B (ratio 1:2 W/W) substrate weight 200 g/L. ***Highly significant $p<0.001$, NS non significant ($p>0.05$), Control without addition.

Table 5. Effect of different peptone concentrations on production of vitamin B_{12} and Folic acid by *Klebsiella pneumoniae.*

Peptone concentration (g/L)	Vitamin B_{12} content (µg/L)	Folic acid content (µg/L)	Substrate consumption (%)
Control	38.74 ± 0.3	45.21 ± 0.2	11 ± 0.0
1.00	43.23 ± 0.4	50.08 ± 1.8	12 ± 0.5
2.00	47.61 ± 0.9	57.11 ± 0.8	13 ± 0.0
3.00	55.09 ± 0.7	60.87 ± 0.3	14 ± 1.0
4.00	57.33 ± 0.5	62.63 ± 0.4	14 ± 1.0
5.00	58.72 ± 0.6	61.91 ± 0.5	13 ± 1.5
P value	***	***	**
F test	556	207	5.5
LSD at 0.05	1.07	1.53	1.54

Initial pH 8. Incubation time 3 days, temp. 30 ^{0}C, substrate W.S+R.B (ratio 1:2 W/W) substrate weight 200 g/L. ***Highly significant $p<0.001$, **Moderate significant *$p<0.01$*, Control without addition.

acid, but non-significant ($p<0.05$) for the substrate.

Peptone

Different concentrations of peptone (1, 2, 3, 4, and 5 g/L) were added to the fermentation medium after being sterilized. The results in Table 5 showed that addition of peptone at concentration 5 g/L to the medium, the best yield of vitamin B_{12} was 58.72 µg/L but the maximum folic acid output was 62.63 µg/L at 4 g/L as well as 14%v substrate consumption. The results of the statistical analysis revealed that the addition of peptone was highly significant ($p<0.001$) for B_{12} and folic yields.

Urea

The investigation was extended to study the effect of different concentrations of urea (0, 5, 1, 2 and 3 g/L) on the production of B_{12} and folic acid. The results presented in Table 6 showed that the best vitamin B_{12} output (47.33 µg/L) was obtained at 0.5 g/L, while, the maximum folic acid output (77.32 µg/L) was obtained at 2 g/L, (Keuth and Bisping, 1993; Herranen et al., 2010). The data of statistical analysis of the addition of urea was highly significant ($p<0.001$) for B_{12} and folicacid.

Conclusion

This study revealed that the productivity of vitamin B_{12} and folic acid was affected by both the carbon and nitrogen sources. The results showed the priority of using glucose and yeast extract which induced highly significant effect on the production process.

Conflict of Interests

The author(s) have not declared any conflict of interests.

Table 6. Effect of different urea concentrations on production of vitamin B_{12} and Folic acid by *Klebsiella pneumoniae.*

Urea concentration (g/L)	Vitamin B_{12} content (µg/L)	Folic acid content (µg/L)	Substrate consumption (%)
control	38.74 ± 0.3	45.21 ± 0.2	11 ± 0.0
0.5	47.33 ± 0.9	46.33 ± 0.2	12 ± 1.5
1	44.12 ± 0.5	59.87 ± 0.7	12 ± 0.5
2	31.23 ± 1.2	77.32 ± 0.8	13 ± 1.0
3	20.23 ± 1.4	69.21 ± 1.1	11 ± 1.0
P value	***	***	NS
F test	390	1199	2.3
LSD at 0.05	1.74	1.28	1.73

Initial pH8. Incubation time 3 days, temp. 30°C, substrate W.S+R.B (ratio 1:2 W/W) substrate weight 200 g/L. ***Highly significant $p<0.001$, NSnon significant ($p>0.05$), Control without addition.

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Full Length Research Paper

Influence of some chemical parameters on decolorization of textile dyes by bacterial strains isolated from waste water treatment plant

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Bacterial species capable of decolorizing textile and laboratory dyes were isolated from textile effluent treatment plant. Two bacterial strains (one Gram negative and another Gram positive bacterial strain) were screened for their ability to decolorize Red H_5BL (reactive dye), Thymol blue (acid dye), Malachite green, Crystal violet (Triphenyl dye) and Congo red (azo dye). The highest decolorization was achieved for Red H_5BL (90%) and lowest for Thymol blue (26%) in 24 h incubation. The effect of different carbon sources and nitrogen sources were studied. The presence of dextrose in the culture medium suppresses the decolorization ability of Gram negative bacterial strain. In case of Gram positive strain, decolorization of Red H_5BL was achieved in the range of 75 to 86% for all the tested carbon sources. During the experiments of checking the effect of carbon sources it was found that shaking culture condition offers high biomass and less color removal of the dye. Decolorization of dyes was effective only under static culture condition. Inorganic or organic nitrogen source has no remarkable effect of decolorization process. Increased dye concentration and salt concentration has negative effect on the process of dye decolorization and biomass synthesis.

Key words: Biodegradation, decolorization, textile dyes, bioremediation.

INTRODUCTION

Textile dyes are of environmental interest because of their widespread use, their potential for formation of toxic aromatic amines and their low removal rate during aerobic wastewater treatment. It is estimated that over 10,000 different dyes and pigments are in common use, with 0.7 million tons of dyestuff being manufactured each year. Two percent of the dye is lost directly in effluent during its manufacturing process while around 10% dye is lost during coloration process (Pearce et al., 2003).

The ability of microorganisms to decolorize and meta-

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bolize dyes has long been known, and the use of bioremediation based technologies for treating textile wastewater has attracted interest (Kothari et al., 2005; Kunjadia et al., 2012). Several physicochemical techniques for textile effluent treatment including adsorption on materials, oxidation, precipitation, photodegradation and membrane filtration have been assessed, but these techniques have been less effective and economically restricted (Mutafov et al., 2007). Many bacterial strains with ability to degrade dyes under aerobic or anaerobic conditions have been isolated from wastewater treatment plant. This indicates that microorganisms may develop the ability of degrading azo components after an adaptation period.

Since microbial populations are responsible for terrestrial nutrient cycling transformations and contribute to the depuration of contaminated ecosystem. Response to stressful conditions may be reflected in change in the size and/or make up of the community, metabolic activity of the microbial biomass, in addition to changes in the taxonomic or functional make up of the community.

Efforts to isolate bacterial culture capable of degrading azo dyes started in 1970 with the reports of *Bacillus subtilis* (Horitsu et al., 1997). Azo dyes were long considered to be nearly non-biodegradable or untransformable by bacteria. Sequential anaerobic and aerobic condition for dye removal has been studied by many researchers. Switching between aerobic and anaerobic metabolic functions during anoxic operation facilitated the reduction of dye to its intermediate in anaerobic condition followed by their mineralization in aerobic condition (Venkat Mohan et al., 2013).

Treatment of textile wastewater is difficult mainly because of complex aeromatic structure and synthetic origin of dyes as well as presence of other organic and inorganic compounds required in dyeing process (Gul, 2013). Bioremediation has to signify the complete microbial breakdown or mineralization of complex material into inorganic constituents such as carbon dioxide, water, mineral components and cell biomass (Alexander and Lustigman, 1996).

In this study, we try to isolate microorganisms, especially bacterial species, capable of decolorization/degradation of various textile dyes present in waste water effluent generated by dyeing and printing industries of our study area. We investigated various levels of chemical and physical parameters that can influence the process of decolorization of selected dyes, in order to design optimum condition for decolorization process.

MATERIALS AND METHODS

Dyes and chemicals

Textile and laboratory dyes were procured from local manufacturer (GIDC, Ahmedabad, Gujarat, India). The dyes were Red H_5BL, Thymol Blue, Malachite Green, Crystal Violet, and Congo red. Red H_5BL dye was used as model for this study. Stock solution of each dye (10 g L^{-1}) were prepared in distilled water and sterilized by passing through 0.45 µm membrane filter. Yeast extract and peptone from Himedia while rest of the all other chemicals used in this study were from Merck.

Screening of organisms and culture condition

Samples collected from different stages of the Common Effluent Treatment Plant (CETP at Jetpur, Gujarat, India) were used for the isolation of bacterial species by enrichment culture technique. The Jetpur town has more than 100 dying and printing industries generating huge quantity of colored waste water. All the waste is carried through drainage line to CETP located 2 Km far from Jetpur. Samples were collected in sample collection bottle and maintained at 4°C during transportation to the laboratory and tested within 24 h. The effluent samples were inoculated in complete medium broth (CMB) containing 100 mg L^{-1} textile dye Red H_5BL. The CMB comprise of Glucose 0.2%; yeast extract 0.5%; Peptone 0.5%; KH_2PO_4 1.0%, $MgSO_4.7H_2O$ 0.02% in 100 ml of distilled water as described by Kothari et al. (2006). The pH of the medium was adjusted to 7. After incubation of 24 h at 30°C, 1 ml aliquot from flask containing effluent as inoculum, were serially diluted and then plated on complete medium agar plate in order to get well isolated colonies for screening of dye decolorizing bacteria (Joe et al., 2008). The pure culture stocks of these isolates were maintained on CM agar slant at 4 °C. CM agar slant comprise of Glucose 0.2%; yeast extract 0.5%; Peptone 0.5%; KH_2PO_4 1.0%, $MgSO_4$ $7H_2O$ 0.02%, and 2% agar agar in 100 ml of distilled water. Bacterial isolates were individually tested for their ability to decolorize dyes in liquid media containing 100 mg L^{-1} textile dye Red H_5BL.

Batch decolorization operation

A loopful of growth from CM agar slant was transferred in to the CMB and incubated at 30°C for 12 h. The cells were in middle of the exponential phase, and used as inoculum with 1 OD at 620 nm. Reaction mixture contained 100 ml CMB in 250 ml flask. 2 ml of the young culture was used as inoculum with 100 mg L^{-1} Red H_5BL dye concentration constant unless until required to change for all experiments. Flasks were incubated in incubator (Remi, RIS-24 BL) in static or shaking condition at 30°C. Periodically samples were withdrawn from reaction flask, centrifuged (Remi, RM-1214) for biomass separation, and supernatant was used to analyze decolorization of dyes using a UV visible spectrophotometer (Shimadzu UV-1800). The growth response of the organism was determined by resuspending the biomass in 2 ml sterile distilled water and OD was taken at 620 nm (Kothari et al., 2005). Decolorization was determined by measuring the absorbance of culture supernatants at the absorbance maxima of the respective dyes. The decolorization assay was calculated as Decolorization activity (%) = (A-B)/ A x 100; where A = initial absorbance and B = observed absorbance (Patil et al., 2008). Parameters of batch operations are summarized in Table 1.

Effect of C and N sources on dye decolorization

Glucose was omitted from the CMB medium and replaced by other carbon sources to be tested. Other carbon sources were Dextrose, Fructose, Galactose, Maltose, Sucrose, Glycerol, Fumeric acid, Starch, Citric acid, Malic acid, Carboxymethylcellulose, and Succinic acid. The combine effect of two carbon sources on dye decolorization was tested by offering combination like Succinic acid + Glycerol, and Succinic acid + Glucose on dye decolorization. These carbon sources were added in the reaction mixture with the 0.5 % final concentration, so in case of combination of carbon source each were added with 0.25 % final concentration. Organic and

Table 1. Summary of parameters of batch operations.

Factors investigated	Culture condition	C source	N source	Dye	Salt (g %)	Initial dye (mg L^{-1})
C source	Static and Shaking	14 different	Peptone	Red H_5BL	0.5	100
N source	Static	Glucose	8 different	Red H_5BL	0.5	100
Dye	Static	Glucose	Peptone	5 different	0.5	100
Salt	Static	Glucose	Peptone	Red H_5BL	0.2 - 3	100
Initial dye	Static	Glucose	Peptone	Red H_5BL	0.5	100 - 500

inorganic nitrogen sources like Peptone, Yeast extract, Ammonium nitrate, Ammonium sulphate, Ammonium tartrate, Ammonium chloride, Sodium nitrate, and Urea were tested for its effect on rate of decolorization of Red H_5BL under static condition. These nitrogen sources were added in reaction mixture with 0.5% final concentration.

Effect of Initial dye and salt concentration on dye decolorization

Various concentrations of Red H_5BL (100, 200, 300, 400, and 500 mg L^{-1}) were prepared in CMB medium and inoculated with stock culture. This experiment was performed in triplicate. The effect of different salt concentration on bacterial growth and its decolorization efficiency were tested by supplementing the reaction mixture with different salt concentrations in the range of 0.2, 0.5, 1, 2, and 3 g%.

Effect of Different Dyes on decolorization

Total of 5 dyes were studied for both the isolated species for their ability to decolorize it. All dyes were added with 100 mg L^{-1} final concentration in individual reaction mixture in three sets of experiment. Samples were collected from reaction mixture, centrifuged, and supernatant was used to analyze dye decolorization by UV-visible spectrophotometer at lambda max of respective dyes (Red H_5BL 512 nm, Thymol Blue 670 nm, Malachite green 622 nm, Crystal violet 588 nm, and Congo red - 505 nm).

RESULTS AND DISCUSSIONS

Screening of bacterial isolates

Experiments to isolate different bacterial species from CEPT effluent resulted in isolation of 33 bacterial species. These strains have been attempted to distinguish on the basis of some morphological, colony characteristics and Gram reactions. The strains have been screened for capability to decolorize dye Red H5BL under laboratory conditions. These strain showed different rates of reduction of color of the dye when grown under stationary batch culture. From these observations, we selected one Gram negative short rod shaped bacterial strain and another Gram positive regular rod shaped bacterial strain for further studies.

Effect of carbon and nitrogen sources on red H_5BL decolorization

In order to check the effect of various carbon sources on the process of dye decolorization by both the bacterial strain, as many as fourteen different carbon sources were tested. Each carbon sources were added at constant concentration in different reaction mixtures. The effect was observed under both the incubation condition – shaking and static condition. Figure 1 showed that there is no marked difference in decolorization pattern when Gram negative bacterial strain was subjected to different carbon sources. It seems that microaerophilic (static incubation) condition favors better decolorization. Isolated Gram positive strain when grown under stationary condition reduced the color of dye Red H_5BL almost completely (80 - 100 %) when grown with all carbon sources except dextrose. When the strain grew under shaking condition, the rate of decolorization was significantly less (4 - 45%). Sharp reduction in decolorization capacity was observed in case of dextrose as a carbon source. Higher concentrations of glucose are known to inhibit dye decolorization by microbial processes (Kumar et al., 2009).

Microbial reduction of dyes is an enzymatic reaction and linked to anaerobiosis because it is inhibited by oxygen. It is possible that non-specific enzymes may be exist in many anaerobic and obligate anaerobic strains to reduce dye under anaerobic condition (Hong and Gu, 2010). Therefore facultative or obligate anaerobes are necessary for azo dye reduction.

Figure 2 explained results of carbon sources effect on decolorization of Red H_5BL by Gram positive bacteria. There was no remarkable difference in decolorization pattern by Gram positive bacteria. This strain also prefer static condition for better decolorization of Red H_5BL, where as shaking condition favors bacterial growth by formation of heavy biomass but negligible dye decolorization was observed.

Organic and inorganic nitrogen sources have influence on the decolorization of various textile dyes under static conditions. Gram negative strain has less effect (57 to 77% decolorization) of nitrogen source whether it is organic or inorganic, where as Gram positive strain showed much influence of organic or inorganic nitrogen sources on decolorization process (43 to 84% decolorization). Results showed that yeast extract favors highest dye

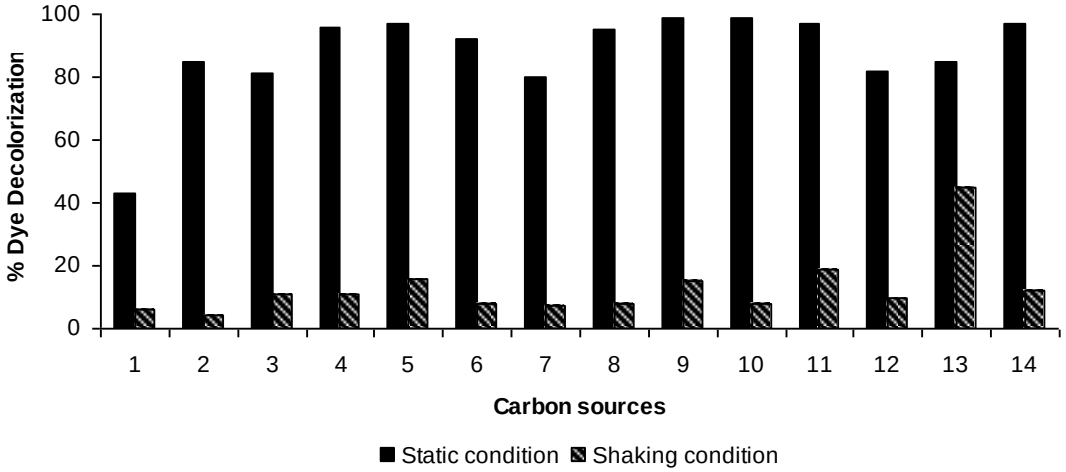

Figure 1. Influence of various carbon sources on decolorization of Red H_5BL by a Gram negative bacteria under static and shaking condition. Numbers on X - axis indicates different carbon sources as; 1. Dextrose, 2. Fructose, 3. Galactose, 4. Maltose, 5. Sucrose, 6. Glycerol, 7. Fumeric acid, 8. Starch, 9. Citric acid, 10. Malic acid, 11. Succinic acid + Glycerol, 12. Succinic acid + Glucose, 13. Carboxymethycellulose, 14. Succinic acid.

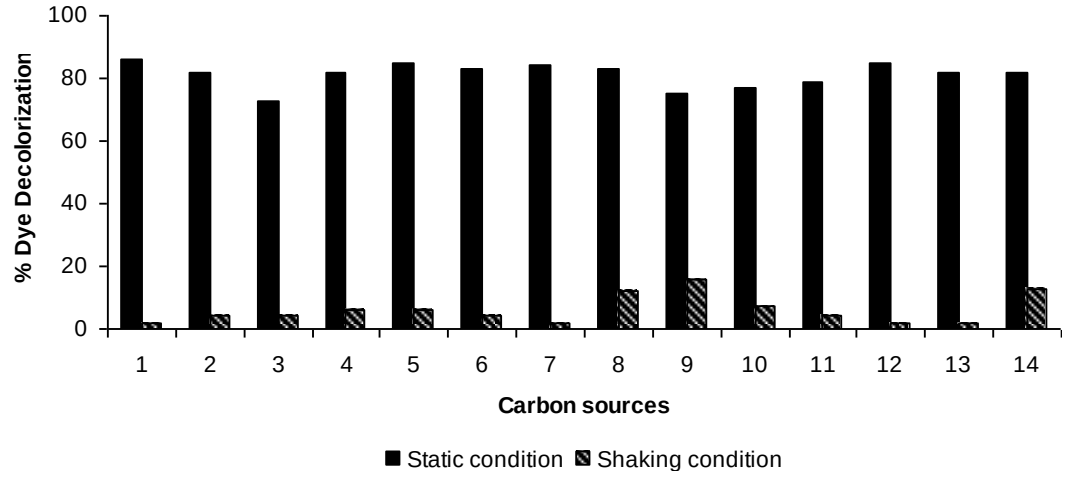

Figure 2. Influence of various carbon sources on decolorization of Red H_5BL by a Gram positive bacteria under static and shaking condition. Numbers on X - axis indicates different carbon sources as ;1. Dextrose, 2. Fructose, 3. Galactose, 4. Maltose, 5. Sucrose, 6. Glycerol, 7. Fumeric acid, 8. Starch, 9. Citric acid, 10. Malic acid, 11. Succinic acid + Glycerol, 12. Succinic acid + Glucose, 13. Carboxymethycellulose, 14. Succinic acid.

decolorization for both the strain; 77 and 84% for Gram negative strain and Gram positive strain respectively (Figure 3).

Effect of different dye on decolorization

Textile industry effluent contains wide variety of dye with different concentration in each batch. Various commercial and laboratory dyes were used to observe the rate of decolorization by both the strains. Both the strains showed highest decolorization of Red H_5BL, 90 and 88% by Gram negative bacteria and Gram positive bacteria respectively (Figure 4). All the three laboratory dyes malachite green, crystal violet, and congo red were decolorized more than 50% by both the strains. The lowest decolorization was of thymol blue (26%) by Gram positive bacterial strain among all tested dyes.

Effect of salt and dye concentration on dye decolorization

Effect of different salt concentration on the growth of the organisms and decolorization was studied by using different sodium chloride concentrations in CMB medium.

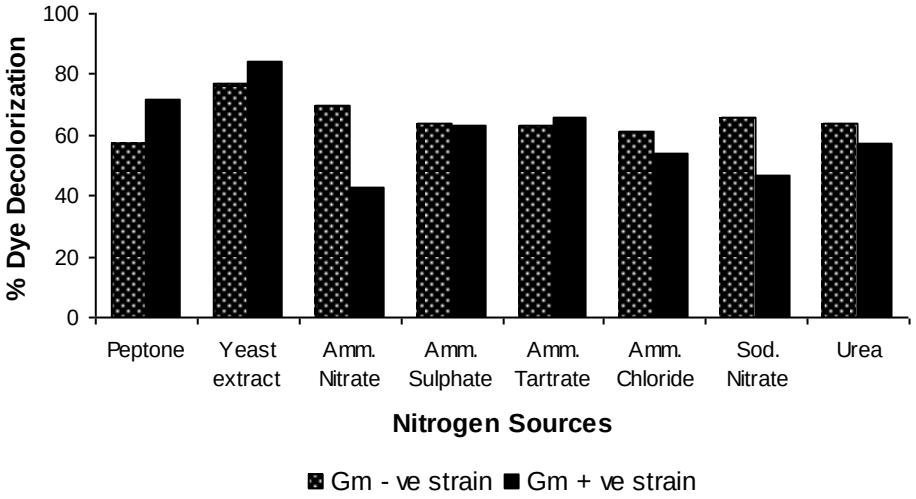

Figure 3. Effect of nitrogen sources on decolorization of Red H5BL by Gram negative strain and Gram positive strain under static condition.

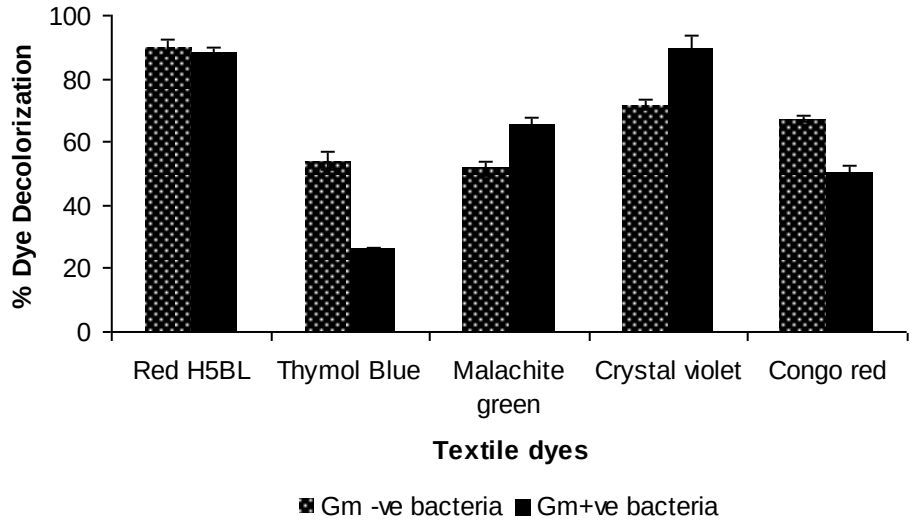

Figure 4. Various textile dye decolorization by both Gram negative strain and Gram positive strain.

Halophilic bacteria have also been reported to decolorize azo dye under high salt condition (Guo et al., 2007). For Gram negative bacterial strain, as the salt concentration was increased from 0.2 to 3 g%, the biomass was reduced by around 54%, while decolorization ability was drastically affected and reduced up to 72%. At high salt concentration, dye decolorization process was affected more than Gram negative bacterial growth (Figure 5). Salt concentration higher than 1 g% found inhibitory to the process. Sharp reduction in biomass and decolorization capacity was observed only after salt concentration higher than it. Almost same pattern of reduction in decolorization efficiency and biomass was observed in case of Gram positive bacteria. The results obtained are indicative of importance of plasma membrane in transport of molecules across the membrane, as at high salt concentration surrounding the cell, Na^+ / K^+ will affect the permeability of the membrane.

The decolorization activity for both the selected organisms was studied using Red H5BL at different initial concentration varying from 100 to 500 mg L^{-1}. Actual textile effluent contains around 200 mg L^{-1} dye concentration (Kumar et al., 2009). The dye concentration up to 200 mg L^{-1} does not affect the biomass synthesis but the 300 mg L^{-1} dye concentration onward, growth was decreased as the concentration of dye was increased in reaction mixture. A kind of regular pattern was observed in reduction of decolorization capacity as increase in initial concentration of dye in case of Gram negative organism (Figure 6). The result obtained from Gram positive

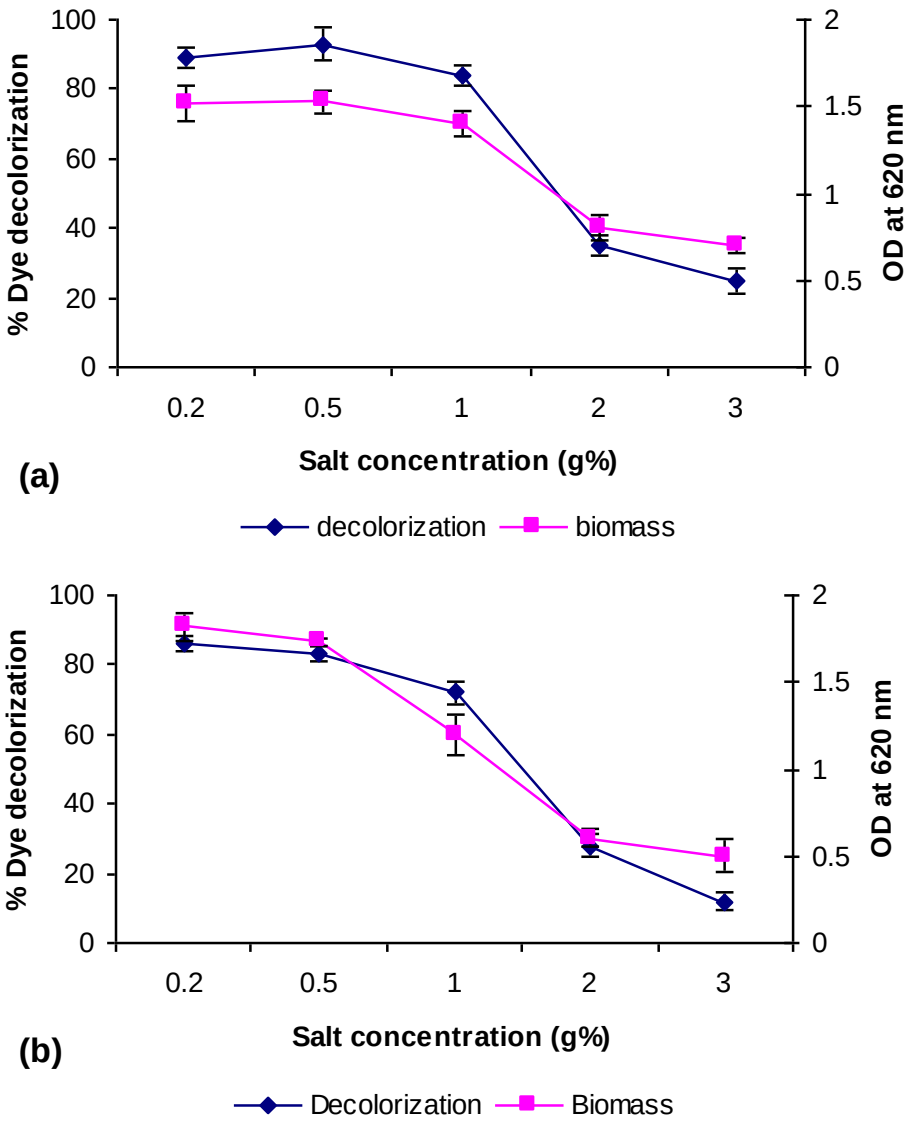

Figure 5. Effect of different salt concentration on bacterial biomass synthesis and rate of Red H_5BL decolorization: **a**. Gram negative bacterial strain, **b**. Gram positive bacterial strain.

organism was slight different from that of Gram negative as there was sharp decline in decolorization activity as well as biomass as the initial dye concentration was increased to 400 mg L^{-1}. As the initial dye concentration increased from 100 to 500 mg L^{-1}, the percentage of Red H5BL decolorization decreased from 90 to 35% for Gram negative bacterial strain and 86 to 12% for Gram positive bacterial strain. In both the cases the increased concentration of dye has inhibited the bacterial growth as evident from results. The decrease in decolorization efficiency might be due to the toxic effect of dyes (Ayed et al., 2009).

Dye containing wastewater treatment presents an arduous task. Wide ranges of pH, salt concentration and variety of chemical structures often add to the complications. Although decolorization is a challenging process to the textile industry, microbial decolorizing system show great potential for achieving total color removal with only hours of exposure.

Conclusion

The bacterial diversity isolated from wastewater effluent plant indicated presence of wide variety of organisms at the contaminated sites. All the isolated organisms showed different decolorization capacity. Both the strains under study showed much similarity in their pattern of Red H_5BL decolorization. No significant effect of carbon and nitrogen sources on dye decolorization process

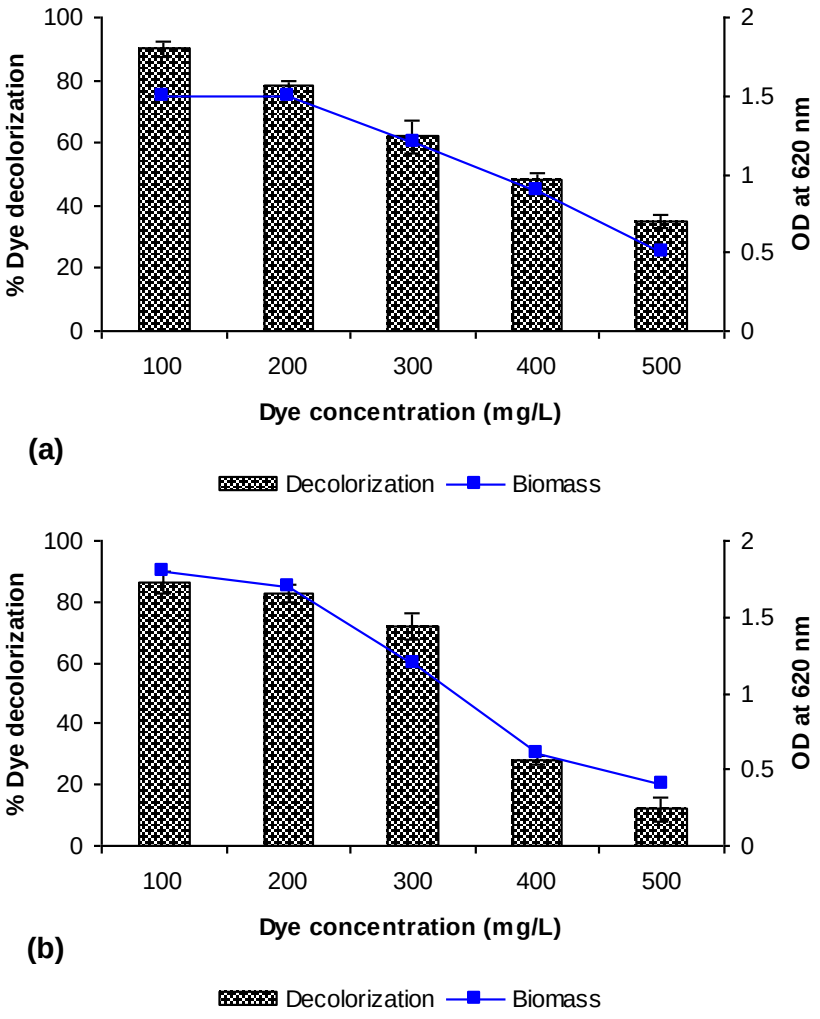

Figure 6. Effect of different initial dye concentration on bacterial biomass synthesis and rate of Red H_5BL decolorization: **a**. Gram negative bacterial strain, **b**. Gram positive bacterial strain.

indicated organism's adaptability to diversified environment. The difference in decolorization capacity of various dyes may be correlated to the difference in the dye structure and its complexity. Their ability to decolorize dye is due to their environment from where they have been isolated. This indicates that microorganisms may develop the ability of degrading azo components after an adaptation period. The Gram negative bacterial strain under study showed more tolerance to high concentration of initial dye concentration than Gram positive strain.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

First characterization of CTX-M-15 and DHA-1 β-lactamases among clinical isolates of *Klebsiella pneumoniae* in Laghouat Hospital, Algeria

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Extended-spectrum β-lactamases (ESBL) are a problem of great concern owing to the potential transmission of resistance to others bacterial species. Our study aims to investigate the ESBL and plasmid-mediated β-lactamases produced by *Klebsiella pneumoniae* strains isolated from 2010 to 2012, among patients hospitalized in "Ahemida Ben Adjila" hospital, Laghouat, Algeria, and to seek a possible clonal dissemination. Antimicrobial susceptibility testing was performed by the agar diffusion method, the characterization of resistance genes to β-lactam antibiotics was performed by PCR amplification and gene sequencing, and molecular typing was performed by ERIC-PCR. In total, of 112 clinical strains of *K. pneumoniae* isolated, nine isolates produced an ESBL. Antibiotics susceptibility testing showed a complete resistance to the majority of third-generation cephalosporins and a very frequent resistance to aminoglycosides and fluoroquinolones resistance. PCR analysis and sequencing showed that all isolates produced ESBL CTX-M-15; three strains of them also produced the cephalosporinase DHA-1. Molecular typing showed that most strains were not related; only three strains that produced CTX-M-15 and DHA-1 had identical profiles suggesting a clonality link. This study revealed the dissemination of ESBL CTX-M-15 and plasmid-mediated AmpC cephalosporinase DHA-1 in *K. pneumoniae*. This is a first report of these enzymes at the Laghouat hospital.

Key words: *Klebsiella pneumoniae*, resistance, CTX-M-15, DHA-1, genotyping, Algeria.

INTRODUCTION

During the past 30 years, extended-spectrum β-lactamases (ESBL) diffused in most Enterobacteriaceae species, especially in *Klebsiella pneumoniae.* Among ESBL CTX-M enzymes, are a major problem because

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Table 1. Sequences of the primers used to detect bla- genes.

Target	Primer	Sequence	Annealing temperatures (°C)	Reference
CTX-M-1	CTXM1 A2 CTXM1 B2	5' - CTT CCA GAA TAA GGA ATC - 3' 5' - CCG TTT CCG CTA TTA CAA - 3'	48	De Champs et al. (2004)
DHA-1	AmpR-AmpcF AmpCR	5' - GGTAAAACTGAGATGACGGGC - 3' 5' - TTATTCCAGCGCACTCAAAAT - 3'	56	Hennequin et al. (2012)
ERIC–PCR	ERIC2	5' - AAG TAA GTGACT GGG GTG AGC G - 3'	64	Dumarche et al. (2002)

they have been involved in many nosocomial outbreaks and are associated with increased mortality (Edelstein et al., 2003; Lebessi et al., 2002; Podschun and Ullmann, 1998). CTX-M β-lactamases enzymes belong to class A. They preferentially hydrolyze cefotaxime more than ceftazidime (Bonnet, 2004). Currently, the CTX-M group 1, particularly CTX-M-15, appear to be more widespread and prevalent in many countries in the world (Carrer and Nordmann, 2011; Lavigne et al., 2007), while other ESBLs of the TEM and SHV subgroups seem to decrease. However, resistance to third generation cephalosporins in *K. pneumoniae* is no longer exclusively due to class A beta-lactamases. The acquisition of AmpC cephalosporinases is associated with a high level of cephalosporin resistance (Hanson, 2003). The earliest report of AmpC beta-lactamases was in 1990, with the identification of MIR-1 beta-lactamase (Papanicolaou et al., 1990). These enzymes are classified in Bush's Group 1 (Ambler's class C). They are generally highly expressed and confer resistance to most β-lactams, except for carbapenems (Philippon et al., 2002). The main objectives of this study were to determine the frequency of resistance to 3rd generation cephalosporins of *K. pneumoniae* strains isolated from patients hospitalized in Laghouat hospital, Algeria, to characterize the β-lactamases involved and to highlight the genetic diversity of these strains.

MATERIALS AND METHODS

Bacterial strains

This is a retrospective study over a period of three years from 1 January 2010 to 31 December 2012. It focused on 112 nonrepetitive *Klebsiella pneumoniae* strains isolated from patients hospitalized in "Ahemida Ben Adjila" hospital, Laghouat, Algeria. All patients hospitalized for more than 48 hours were included in the study. The samples correspond to different colonization sites (urine, catheters, rectal swabs and pus), and essentially isolated from services: Women Medicine; General Surgery, Orthopedics, Pulmonology and Intensive Care Unit. Some epidemiological data such as age, gender and origin of service were recorded in the first time for each patient. Species' biochemical identification was performed using the API 20E® identification system (bioMérieux, Marcy l'Etoile, France).

Antimicrobial susceptibility and synergy testing

The antibiotics susceptibilities were determined on Mueller–Hinton agar by the standard disk diffusion procedure as described by the Antibiogram Committee of the French Society for Microbiology (CASFM, 2010). The following antibiotics were tested: Amoxicillin (25 µg), amoxicillin/clavulanic acid (30 µg), ticarcillin (75 µg), ticarcilline/clavulanic acid (85 µg), piperacillin (75 µg), piperacillin + tazobactam (85 µg), cephalotin (30 µg), cefuroxime (30 µg), cefixime (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefepime (30 µg), cefpirome (30 µg), imipenem (10 µg), aztreonam (30 µg), cefoxitin (30 µg), gentamicin (15 µg), tobramycin (10 µg), amikacin (30 µg), nalidixic acid (30 µg), ofloxacin (5 µg), ciprofloxacin (5 µg), kanamycin (30 µg), fosfomycin (50 µg), tetracycline (30 µg), chloramphenicol (30 µg), sulfonamide (200 µg), netilmicin (30 µg), trimethoprim (5 µg), sulphamethoxazole/trimethoprim (25 µg), colistin (50 µg), and ceftazidime/clavulanic acid (30/10 µg). The antibiotic disks were obtained from Oxoid, England.

All isolates were phenotypically screened for the production of ESBLs using the double-disk synergy test (DDST) (Jarlier et al., 1988). *Escherichia coli* ATCC 25922 was used as a control strain.

Analytical isoelectric focusing (IEF)

IEF was performed to determine the isoelectric point of the different β-lactamases of each strain as previously described by Bonnet and coll (Bonnet et al., 2000). β-lactamases with known pI were used as standards: CTX-M-1 (pI 8.4), CTX-M-14 (pI 7.9), CTX-M-15 (pI 8.6).

β-Lactamase characterization

The DNA extraction was conducted by preparing a suspension of the strains to be studied in 200µl of distilled water. After boiling for 10 min of suspension and centrifugation for 7 min at 13, 000 × g, and the supernatant was collected in a new 1.5 ml Eppendorf tube and stored at -20°C.

The detection of genes encoding the β-lactamase ESBL and plasmid-mediated AmpC cephalosporinases (DHA-1) was performed by Polymerase Chain Reaction (PCR), the operating conditions and primers used were described in Table 1. The PCR reactions mixture consists of: 5 µl of each primer (10 picomoles/µl), 1 µl of dNTP, 10µl of PCR reaction buffer 5X and 0.25 µl of Taq DNA polymerase (Promega). The DNA was amplified in a final volume of 50 µl, in a thermocycler: either Biometra T Personal (Labgene) or the Primus 96 plus (Biotech). The PCR products were separated in 1% agarose gels. When PCR was positive, the amplicon was purified and sequenced in GATC Biotech AG (European Custom Sequencing Centre, Gottfried-Hagen-Straße 20, 51105 Köln), to identify precisely the desired β-lactamases.

Table 2. Characteristics of extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* isolates.

Isolate	Wards	Date of isolation (day/month/year)	Sample origin	Sex	Age (years)	β-lactamase pI	β-lactamase gene
Kp3	Orthopedics	13/02/2010	Pus	F	32	5.4 + 7.5 + 7.7 + 8.6	CTX-M15
Kp9	Orthopedics	19/07/2010	Rectal	F	85	5.4 + 7.5 + 7.7 + 8.6	CTX-M15
Kp18	Women Medicine	04/03/2012	Rectal	F	70	5.4 + 7.5 + 7.7 + 7.8 + 8.6	CTX-M15, DHA-1
Kp20	Pulmonology	12/03/2011	Urine	H	60	5.4 + 7.7 + 8.6	CTX-M15
Kp31	General Surgery	22/05/2011	Rectal	F	27	5.4 + 7.7 + 8.6	CTX-M15
Kp51	Intensive care unit	04/09/2011	Rectal	F	49	5.4 + 7.7 + 8.6	CTX-M15
Kp55.2	Women Medicine	25/04/2012	Pus	F	29	5.4 + 7.7 + 7.8 + 8.6	CTX-M15, DHA-1
Kp57	Intensive care unit	07/08/2012	catheters	H	30	5.4 + 7.5 + 7.7 + 8.6	CTX-M15
Kp73	Intensive care unit	29/11/2012	Rectal	F	39	5.4 + 7.5 + 7.7 + 7.8 + 8.6	CTX-M15, DHA-1

Conjugation transfer experiments

Conjugation experience was performed with *E. coli* C600 Rif R (resistant to rifampicin) as the recipient strain. The strains were inoculated into a brain-heart infusion broth (BHIB) and incubated overnight at 37 C°. The transconjugants were selected on Mueller-Hinton agar containing rifampicin (300 µg/L) and cefotaxime (1 µg/L). After, the transconjugants pushed into the selection boxes were tested vis-à-vis the antibiotics to detect the presence of resistance phenotype towards 3rd generation cephalosporins and cefoxitin.

Enterobacterial repetitive intergenic consensus PCR (ERIC-PCR)

The total DNA of each strain was tested by ERIC-PCR (Repetitive enterobacterial intergenic consensus). For ERIC-PCR reaction, DNA was amplified using the only one primer ERIC-2: 5' AAGTAAGTGACTGGGGTGAGCG 3' (Table 1), with the following program: 5 min at 94°C and 1 min at 36°C, then 36 cycles of 3 min at 72°C, 1 min at 94°C, and 1min/30 s to 36°C, and ending with 10 min at 72°C.

The preparation conditions of ERIC-PCR mix were as follows: distilled H_2O: 28.4 µL, dNTP (Eurogentec): 3.2 µl, $MgCl_2$: 3 µl, 5X Buffer: 5 µl, eric2 Primer: 5 µl, Go taq (Promega): 0.4 µl. After, 5µl mixed was added to the DNA crude extract diluted to 1/100 th with sterile distilled water. Fingerprints were visually compared and the patterns differing by at least one amplification band were classified as different.

RESULTS

During the period of our study, 215 patients were included and 112 strains of *Klebsiella pneumoniae* were isolated. The double synergy test was positive for nine strains of *K. pneumoniae,* suggesting the probable production of ESBL. These nine strains produced extended-spectrum β-lactamases (ESBLKp) (frequency of 8%). The majority of patients were women (78%) and their ages ranged from 27 to 85 years. Some hospital services appeared more concerned with the problem of resistance due to ESBL production, including the intensive care unit of which 33% of cases were noticed, followed by the orthopedics and women medicine service with 22% for each (Table 2).

The nine isolates tested were resistant to extended-spectrum cephalosporins, amoxicillin, ticarcillin, amoxicillin/clavulanic acid and ticarcilline/clavulanic acid. Only 33% of the strains were susceptible to piperacillin-tazobactam. In addition, three strains resistant to cefoxitin. For these three strains induction was observed between imipenem and third generation cephalosporins, suggesting the production of an inducible céphalo-sporinase. All strains remained susceptible to imipenem (Figure 1).

The ESBL phenotype is associated with resistance to aminoglycosides: kanamycin (100%), tobramycin (100%), gentamicin (78%), and amikacin (55%); sulfonamides (100%), and fluoroquinolones (89%). All strains were susceptible to colistin and fosfomycine. The determination of the isoelectric point (pI) showed that the nine strains produced β-lactamases of different pI: 5.4 (compatible with a TEM-1-type penicillinase), 7.5 (compatible with a OXA-1-type oxacillinase), 7.7 (compatible with the chromosomal penicillinase SHV-1) and 8.6 (compatible with a CTX-M-type ESBL). In addition to these β-lactamases, the three strains Kp18, Kp55.2 and kp73 also produced one β-lactamase of a pI: 7.8 (Figure 2).

Molecular characterization of β-lactamases by polymerase chain reaction (PCR) and sequencing revealed that the nine strains of *K. pneumoniae* producing ESBL from hospitalized patients in different departments in the hospital carried the same gene $bla_{\text{CTX-M-15}}$. Moreover, we noticed the presence of plasmid-mediated AmpC cephalosporinase by $bla_{\text{DHA-1}}$ gene in three strains Kp18, Kp55.2 and kp73.

Following the conjugation, antimicrobial susceptibility of the nine transconjugants showed resistance towards 3rd generation cephalosporins with the presence of synergy,

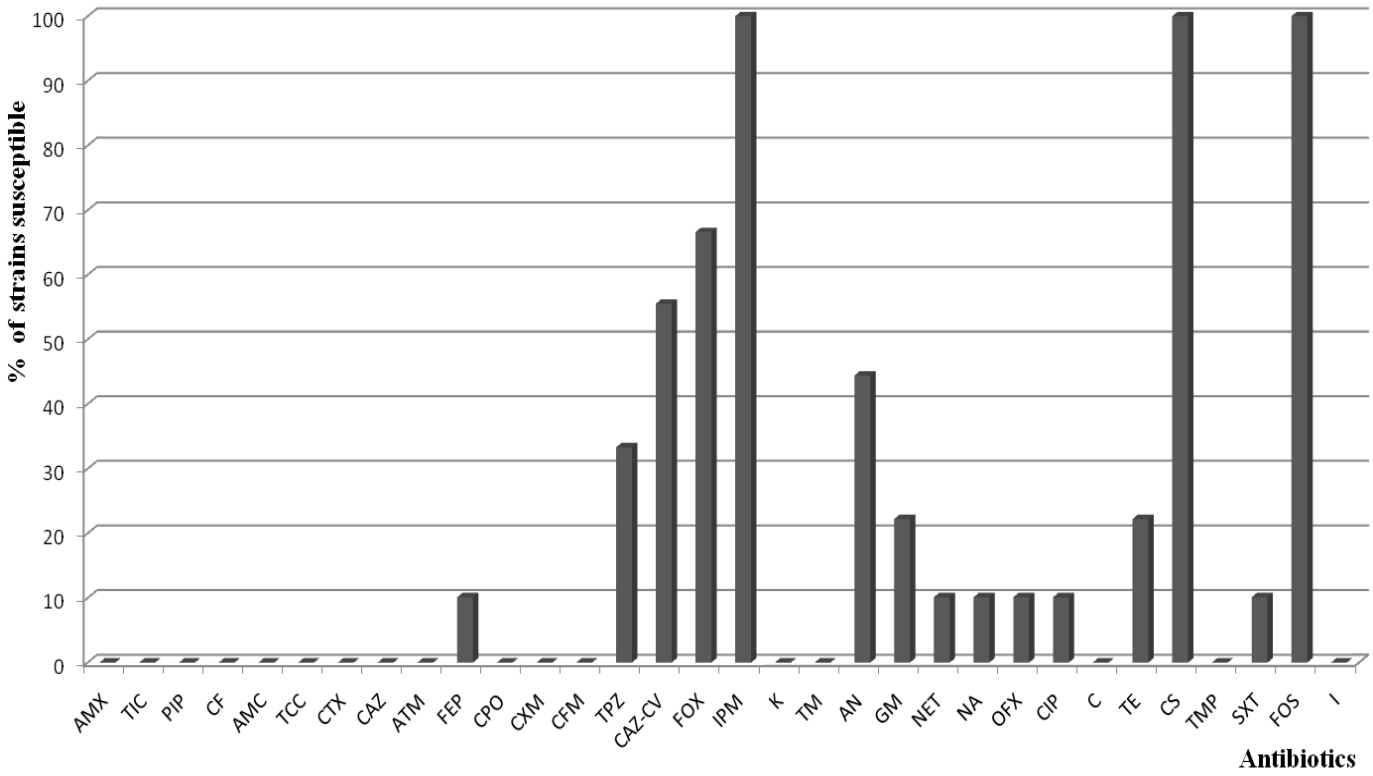

Figure 1. Susceptibility to antimicrobials of ESBL-producing *Klebsiella pneumoniae* isolates. AMX: amoxicillin, TIC: ticarcillin, PIP: piperacillin, CF: cefalotin, AMC: amoxicillin/clavulanic acid, TCC: ticarcilline/clavulanic acid, CTX: cefotaxime, CAZ: ceftazidime, ATM: aztreonam, FEP: cefepime, CPO: cefpirome, CXM: cefuroxime, CFM: Cefixime, TPZ: piperacillin + tazobactam, CAZ-CV : ceftazidime/clavulanic acid, FOX: cefoxitin, IPM: imipenem, K: kanamycin, TM: tobramycin, AN : amikacin, GM: gentamycin, NET: netilmicin, NA: nalidixic acid, OFX: ofloxacin, CIP: ciprofloxacin, C: chloramphenicol, TE: tetracycline, CS: colistin, TMP: trimethoprime, SXT: sulphamethoxazole/trimethoprim, FOS: fosfomycin, I: sulfonamide.

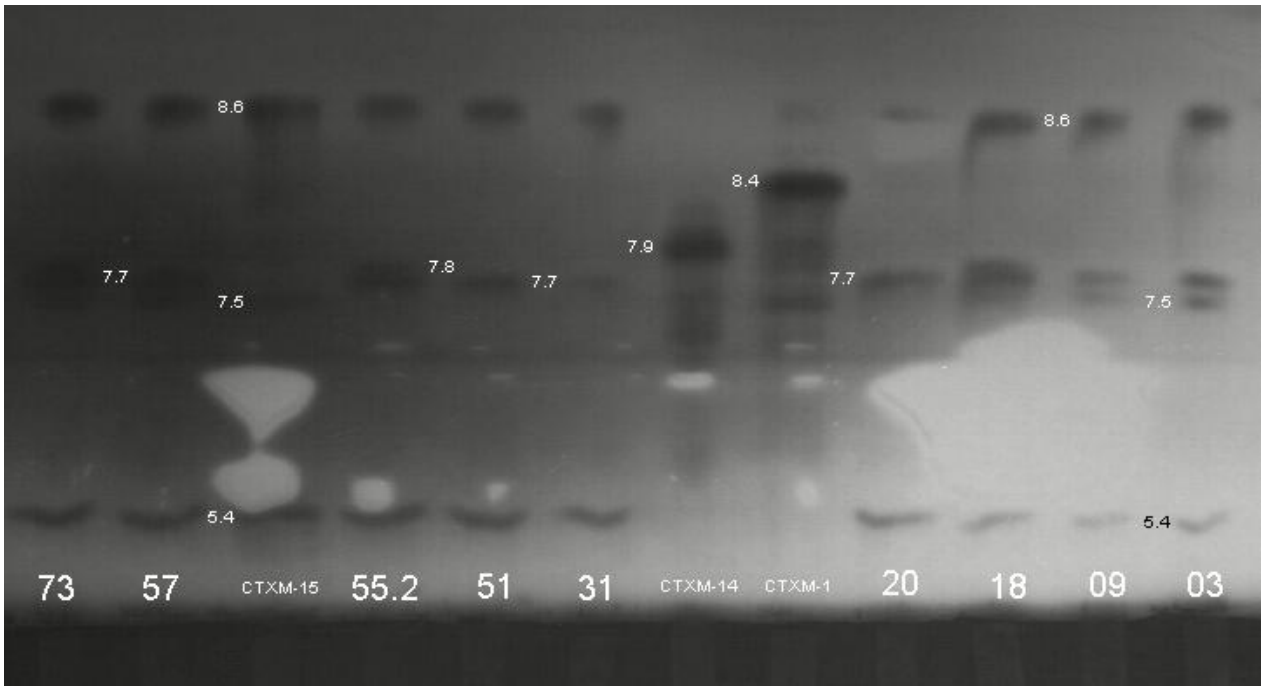

Figure 2. The isoelectric point pI of the extended-spectrum β-lactamase-producing *Klebsiella pneumoniae.*

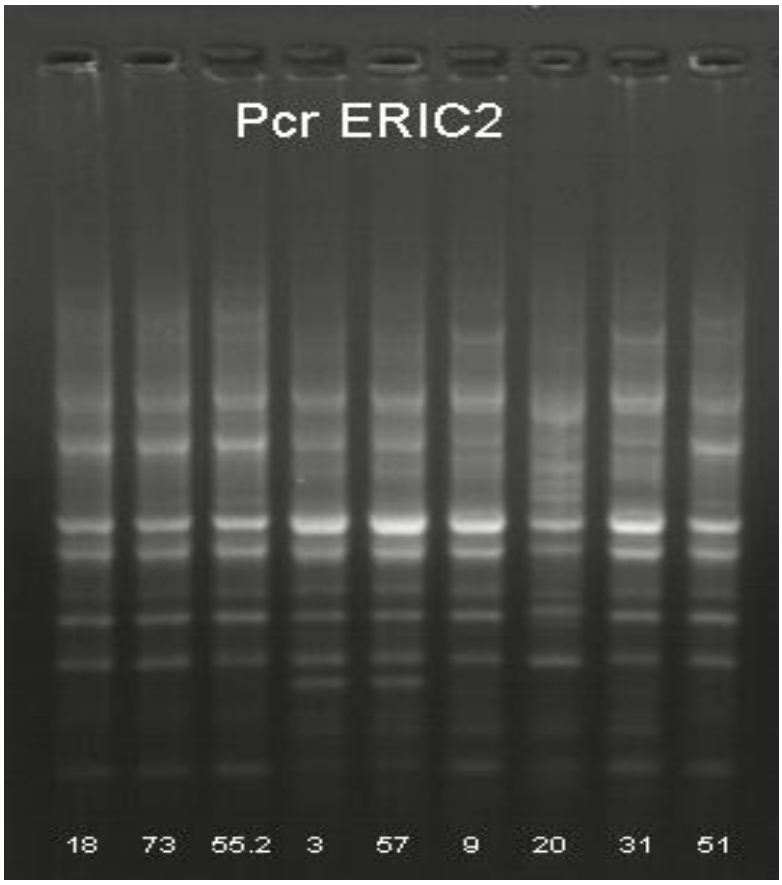

Figure 3. ERIC-PCR profiles of the extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* obtained with the primer ERIC-2.

aminoglycosides, kanamycin, and fluoroquinolones. Thus, the resistance to céfoxitine was observed in 3 transconjugants. This explained that the gene $bla_{CTX\text{-}M\text{-}15}$ and the gene $bla_{DHA\text{-}1}$ gene have been carried by a plasmid, and transferred to the transconjugants by conjugation. Molecular typing by ERIC-PCR showed the presence of seven different ERIC profiles. One profile was common to the 3 strains co-producing CTX-M-15 and DHA-1, suggesting a clonal relatedness (Figure 3).

DISCUSSION

In our study, the prevalence of ESBL producing *Klebsiella pneumoniae* isolates was 8%. The overall prevalence of ESBL production varied considerably according to geographic areas of countries and the different hospital structures. ESBL prevalence rate in this study is still lower compared to those reported in northern Algeria (19.9%) (Messai et al., 2008) and Tunisia (20.2%) (Ben Haj Khalifa and Khedher, 2009). However, higher prevalence rates of ESBL produced by *Klebsiella* were detected in South America (45.4% to 51.9%) (Villegas et al., 2008) and Saudi Arabia (55%) (Al-Agamy et al., 2009). In Pakistan, the prevalence of ESBLs produced in *K. pneumoniae* reached a very high alarming rate with 70% (Shah et al., 2004).

The antibiotics susceptibility profile of the nine strains studied showed resistance to most β-lactams tested except cefoxitin (30% of strains were resistant), and imipenem remained active in all strains studied. Thus, cross-resistance is observed with aminoglycosides and fluoroquinolones, and this could be related to the misuse of broad-spectrum antibiotics (penicillins, cephalosporins, chloramphenicol, tetracyclines, fluoroquinolones, and aminoglycosides).

The proportions found by Ben Haj Khalifa and Khedher in a study of ESBLs produced in uropathogen *Klebsiella* spp, isolated in Tunisian university hospital, are closer to those we observed (100% to 3rd generation cephalosporins, 92.5% to gentamicins, and 67.5% to fluoroquinolones) (Ben Haj Khalifa and Khedher, 2009).

It is now proven that the use of antibiotics, especially 3rd generation cephalosporins for therapeutic purposes, is the most important risk factor in the development of bacterial resistance (Rubin and Samore, 2002). It has become a major public health problem.

We found, the same ESBL CTX-M-15 in the 9 ESBLKp

strains studied. Currently, the ESBL CTX-M are the most frequently isolated in western and eastern Algeria (Ahmed et al., 2012; Nedjai et al., 2012), Tunisia (Elhani et al., 2011), Portugal (Mendonça et al., 2009), China (Liu et al., 2009), in Taiwan (Shu et al., 2010) and many countries worldwide.

In our study, the phenotype of the DHA-1-producing strains was characterized in three cefoxitin-resistant strains, two strains (Kp18, Kp55.2) were isolated in the women medicine service, and one strain (kp73) was isolated in the intensive care unit (ICU).

These enzymes have been recently detected among Enterobacteriaceae such as *E. coli*, *K. pneumoniae*, *Klebsiella oxytoca*, *P. mirabilis* and *Salmonella* spp. These plasmid-mediated cephalosporinases are genetically very close to chromosomal AmpC cephalosporinases, and the transfer of the genes coding these enzymes requires mobile genetic elements (Philippon et al., 2002; Bush et al., 1995).

Our results are comparable with those of other studies in Spain, China, and France, which observed the diffusion of DHA-1 plasmid-mediated cephalosporinases in *K. pneumoniae* strains (Hennequin et al., 2012; Guo et al., 2012; Tobes et al., 2013). There are few studies concerning the cephalosporinases AmpC diffusion in this species in Algeria. Only one study was conducted in three hospitals of Algiers, where there is the detection of the production of cephalosporinases CMY-2 in *K. pneumoniae* and DHA-1 was detected in *Enterobacter cloacae* strains (Iabadene et al., 2009). So, this is the first detection of DHA-1 in *K. pneumoniae* in Algeria.

Typing ESBL by ERIC-PCR showed a single profile for the three strains producing CTX-M-15 and DHA-1, and a genetic diversity for other strains that produced only CTX-M-15. This suggests that the distribution of DHA-1 is the result of the spread of a single clone among patients in the two services: Women Medicine and Intensive Care Unit, hospital.

The acquisition of the gene blaCTX-M-15 by strains of *K. pneumoniae* probably occurred by horizontal transfer from strains of *E. coli*. Several studies have described that the gene *bla*CTX-M-15 has been carried by transferable plasmids. A study in Spain showed that the gene blaCTX-M-15 is carried by the same plasmid with a size of 180kb (Valverde et al., 2008). On the other hand, in another study, the size of plasmid was 150kb, on the basis of which it is associated with genes encoding for aminoglycosides resistance (Mesko et al., 2009).

Our study is the first report made to "Ahemida Ben Adjila" Laghouat Hospital, Algeria. It allowed us to reveal the distribution of β-lactamase CTX-M-15 and DHA-1 in *K. pneumoniae* strains which produce the extended-spectrum β-lactamases, with the first detection of plasmid-mediated cephalosporinases DHA-1 produced by *K. pneumoniae* in Algeria.

Nowadays, controlling antibiotics use is essential to monitor the increasing spread of genes resisting to antibiotics.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Postharvest fruit spoilage bacteria and fungi associated with date palm *(Phoenix dactylifera L*) from Saudi Arabia

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Date fruits are consumed as traditional and ideal food in Saudi Arabia. It provides a wide range of essential nutrients and potential health benefits. Twelve (12) most consumed varieties of date fruits and their seeds available in the open markets of Riyadh, Medina and Kharj were screened for the presence of bacteria and fungi. Our study reveals that these fruits carry a heavy load of both fungal and bacterial pathogens. The variety Sukhari was found to be the most contaminant fruit with fungi while the bacterial contamination was highest in the variety Mabroon. The Genus *Aspergillus* was represented by seven species, amongst which *Aspergillus* niger was the most predominant fungi. Potential pathogens like *Staphylococcus aureus* and *Escherichia coli* were isolated besides six species of the genus *Bacillus.* Fruits were more contaminated than their seeds.

Key words: Date fruits, seed borne, fruit spoilage, open markets.

INTRODUCTION

The date palm *(Phoenix dactylifera L*) is one of the oldest fruit trees of the world and is closely associated with the life of the people in the Middle East including the Kingdom of Saudi Arabia since ancient times. It has religious values as well as cultural importance. In Saudi Arabia, date palm is the most important cash and fruit crop grown in different regions. It produces yearly about 900 thousand tones and ranks as the third largest producer in the world (FAO, 2008). Saudi Arabia, ranked number one among the date producers and exporting countries in the world, where it produces 7170 tons of date fruits annually (Al-Showiman and BaOsman, 1992). Most of the produce in Saudi Arabia is consumed locally as human food, while the rest is exported. Saudi Arabia is also a genetic centre of date-palm trees and there are more than 400 different cultivars of fruiting date palm of economic value (Fayadh and Al Showiman, 1990).

Date fruits contain a high percentage of carbohydrate (44.88%), protein (2.3-5.6) and dietry fiber (6.4-1.5%). The fat percentage reach upto (0.2-0.5%), including 14 types of fatty acids (Al Kahtani et al., 2011). Date fruits are consumed in many forms and at all stages of the fruit development that is Kimri, Rotab and Tamr. In addition to the direct consumption of the fruit, dates are also utilized in many ways in modern industries (Mustafa et al., 1983; Sawaya et al., 1989; Shinwari, 1993) Therefore special attention is being paid by the government of Saudi Arabia in providing subsidies and price support of low grade varieties (Al Shuaibi, 2011).

In practice, besides bacteria and yeast, molds are

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considered to be the major causative agents of the spoilage of date fruits at all stages of ripening on trees,as well as during storage and processing. Some potential pathogens and mycotoxin producing microbes isolated from date fruits include *Staphylococcus aureus, Escherichia coli*, species of *Aspergillus, Penicillium, Alternaria* and *Fusarium* (Kader, 2007; Hamad, 2008; Hayrettin et al., 2012; Aido et al., 1996, El-Sherbeeny et al., 1985; Abdulsalam et al., 1991).

Our study aims at enumeration of bacteria and fungi, causing spoilage of date fruits and seed borne pathogens from fruits sold as loose dates, in the open markets of Saudi Arabia .It also highlights the risks and awareness of consumption of contaminated fruits which carry mycotoxin producing fungi and potential pathogens. To our knowledge this is the first time that fruit varieties that is Ajwa, Amber, Rabeae and Mashrooq are being screened in Saudi Arabia for the presence of microbes responsible for causing fruit spoilage and also the first report of identification of isolated bacterial strains upto species level.

MATERIALS AND METHODS

Date fruits grown locally in Saudi Arabia, sold as loose dates in open markets were chosen for the study. Date fruits were collected from open market of Riyadh, Medina and Al Kharj. The varieties choosen were Ajwa, Amber,Barhi, Mashrooq ,Mabroon, Rabeae , Al Segae, Khodary,Khalas, Naboot Sultan, Naboot-Seif and Sukhari .

Isolation of Seed-Borne Fungi

Isolation of seed borne fungi was carried out by Standard blotter method as described by the International Seed Testing Association (ISTA 1976). A total of 50 seeds from each variety were screened. Five seeds per plate from each variety were plated in 13 cm diameter Petri dishes over moist filter paper and Potato dextrose agar (Oxoid ,UK). The paper was kept moist throughout the experimental period by adding sterile water. Ten replicates were used for each variety. These plates were then incubated at 25±2°C for one week and cultured on potato dextrose agar (Oxoid, UK) for identification.

Isolation of Fungi from date fruits

Dates fruits were cut into approximately 2 cm^3 pieces and five pieces were placed aseptically on Potato Dextrose Agar (PDA Oxoid, UK) and moist filter papers, incubated at 25±2°C for 5-10 days. Mixed cultures were purified using single spore and maintained on PDA.Ten replicates were used for each variety.

Identification of fungi

Identification of the purified cultures was done either to generic or the species level according to their appropriate taxonomic key and description (Ellis, 1971; Raper and Fennell, 1965; Ramirez, 1982; Nelson et al., 1983). Pure cultures of the isolated fungi were maintained at 5°C.

Bacterial isolation

Fruits flesh was washed and deseeded; seeds were washed with sterile distilled water, then rinsed with chlorinated water (2%) for 1 min and then washed off four times again with sterile distilled water, microbial loads were calculated both from seeds and flesh. Sterilized date fruit samples (10 g) were aseptically weighed into sterile stomacher bags (Seward Medical , London ,U.K) with 90 ml sterile 0.1% peptone water (Oxoid CM9). Samples were then homogenized for 2 mins and a serial dilution 1.1000 were prepared. Aliquots (0.1 ml) of diluted homogenate were then plated on Nutrient Agar plates (N.A, Oxoid) and Plate Count Agar (PCA, Oxoid) in triplicates, plates were incubated at 37° C for 24 h for bacterial enumeration. The mean of the bacterial load obtained from the plates was reported as number of bacteria /plate/ml on different media Nutrient agar (N.A, Oxoid) and Plate Count Agar (PCA, Oxoid).

Same procedure as fruits was applied for isolation for seed borne bacteria, besides sterilized seeds were placed (5 per plate) on N.A (Oxoid). Plates were incubated at 37°C and examined every 24 h for up to 5 days .Bacterial isolates were randomly selected from outgrowths on and around seeds and streaked on N.A and glucose peptone agar until pure cultures were obtained for characterization and Identification (Collins et al., 2004).

Identification of bacterial isolates

Identification of bacterial isolates obtained from date fruit varieties (both flesh and seeds) in our study, were characterized and identified by their cultural morphology and biochemical properties (Collins_et al .,2004; Cheesebrough, 2000) tentataively. However, further confirmation for gram positive rods was done with the help of API 50 CH/ B galleries while two isolates which were different from the above isolates were identified with the help of morphologyical charactertics and biochemical test alone. Pure cultures of isolates were stored in nutrient broth (N.B, Oxoid) containing 15% glycerol (Merck) at -4°C. Among all the bacteria isolated, only those gram positive, endospore forming rod producing bacteria were selected for further identification according to their carbohydrate fermentation using the API 50 CH/ B galleries at least in duplicate.

Results were recorded after 24 hof incubation at 37°C. The ultimate identification was ensured by the API web identification software (Biomerieux, France) as indicated by the manufacturer's instruction.

Bacterial Identification by API 50 CH/B and Biochemical tests

The phenotypic characterization of those isolates described as gram positive endospore forming long rod obtained, were identified upto genus level as *Bacillus*. Further biochemical identification were performed by the API 50 CH/B medium.

RESULTS

All cultivars of date fruit were contaminated with fungi whereas bacterial population was found in varying numbers and were absent totally in some date fruit varieties.

Table 1. Percentage occurrence of fungi associated with fruit spoilage of different varieties of date palm.

Fungi isolated	Date varieties screened											
	Ajwa	Amber	Barhi	Khalas	Khodary	Mabroon	Mashrooq	Sukhari	Segae	Naboot Sultan	Naboot Seif	Rabeae
Alternaria alternate	52	68	80	88	72	58	40	28	18	12	84	68
Aspergillus ellipticus	…	4	…	4	…	…	…	…	…	…	12	….
A. flavus	50	54	68	52	42	48	44	38	42	34	42	48
A.fumigatus	….	20	30	6	…	8	…	4	…	18	48	24
A.nidulans	…	22	28	54	24	4	…	4	2	….	….	…
A. niger	80	90	88	82	78	76	80	88	66	86	92	82
A.terreus	54	4	42	52	24	14	18	10	18	12	58	54
Aspergillus sp.	…	2	…	…	..	4	2	…	…	…	2	…
Bipolaris sp	….	…	28	20	14	12	34	...	10	8	14	4
Cladosporium cladosporoides	…	…	4	22	8	…	6	6	8	…	16	…
Curvularia lunata	2	6	12	34	28	18	4	2	10	6	38	20
Fusarium monoliforme	…	…	28	38	52	32	14	16	8	6	44	...
F.oxysporum	20	54	34	54	68	42	52	22	28	54	66	52
F.solani	20	18	8	12	20	4	8	12	20	14	28	14
Mucor spp	…	…	4	…	…	…	…	2	….	6	4	….
Penicillium chrysogenum	54	58	80	78	34	32	20	6	82	52	84	82
P.digitatum	…	8	14	32	2	2	4	…	…	…	12	20
P.expansum	…	…	12	2	…	4	8	2	4	…	6	8
Penicillium sp	…	…	6	8	…	4	…	…	…	…	2	…
Rhizopus stolonifer	20	30	74	82	44	14	10	4	6	24	80	72

…, not detected.

Fruit spoilage mycoflora

A total of 20 species belonging to nine genera were isolated from the flesh of date palm fruits used in our experiment (Table 1). The following fungi were isolated from all the cultivars screened, amongst them *Aspergillus niger* (81%) was the most predominant species followed by *Alternaria alternate* (55.66%), *Penicillium stolonifer* (55.16%), *A. flavus* (46.83%), *Fusarium oxysporum* (43.83), *F. solani* (34%) *Rhizopus stolonifer* (38.33%), *Aspergillus terreus* (30%), *F.monoliforme (19.83), Curvularia lunata (15%).* Amongst all the nine genera isolated from the date fruits screened , seven species belonged to the genera *Aspergillus* ,four species belonged to *Penicillium,* three of *Fusarium* and one species each belonged to the genus *Alternaria, Bipolaris, Cladosporium, Curvularia* ,*Rhizopus* and *Mucor.*

Our results reveal that Sukhari, Khalas ,Barhi, Segae and Amber were the most suspectible cultivar compared to the other cultivars. *A. niger* was found in high percentages in variety Sukhari (92%), Amber (90%) and Barhi (88%). A maximum of 700 and 32 isolates were isolated from Sukhari followed by 720 from Khalas and 666 isolates from the cultivar Barhi.

Seed borne fungi

Seed from different cultivars of date palm harboured a total of nine genera belonging to 13 species. *A. niger* was the most predominant followed by *F. oxysporum*, *F. solani, A. flavus, A. alternate* and *R. stolonifer* (Table 2). Seeds from

Table 2. Percentage occurrence of seed borne fungi associated with different varieties of date palm.

Fungi isolated	Date varieties screened											
	Ajwa	Amber	Barhi	Khalas	Khodary	Mabroom	Mashrookh	Sukhari	Sagaei	Naboot Sultan	Naboot Seif	Rabeae
Alternaria alternata	4	6	12	14	10	2	….	16	4	2	14	4
A.carbonarius	…	…	4	…	…	…	…	…	…	2	…	…
Aspergillus flavus	14	22	18	40	42	10	12	18	8	14	40	38
Aspergillus niger	24	42	28	36	26	42	36	24	22	38	48	58
Bipolaris sp	…	…	8	…	…	…	…	…	…	…	18	…
Chaetomium sp	…	…	…	…	14	…		…	…	…	12	4
Dreschlera sp	…	…	8	…	…	4	…	…	…	…	16	
F.oxysporum	12	22	28	60	38	18	14	10	16	12	68	60
F.solani	26	34	18	30	34	24	14	2	38	26	20	28
Penicillium chrysogenum	…	2	8	…	…	4	…	12	10	…	6	…
Rhizopus oryzae	4	…	2	…	…	2	…	…	…	6	…	…
Rhizopus stolonifer	18	8	14	6	…	…	…	…	…	8	10	4
Trichoderma virde	…	…	2	…	…	2	…	…	…	…	4	…

…., Not detected

all the date fruit varieties screened showed the presence of fungi in varying numbers .However *A. niger* , *F. oxysporum* , *F. solani* and *A. flavus* were isolated from all the cultivars screened. *Bipolaris* sp was reported only from the cultivar Sukhari and Barhi while *Chaetomium* was found in Khodary,Sukhari and Segae.

Bacterial contamination of fruit and seed of date palm

Among a total of 30 bacterial isolates form both flesh and seeds of date fruits only 25 could be identified of which all were gram positive rods as confirmed by API and the other two were *S. aureus* and *E. coli* (Table 3). API ensured that most isolates belong to the genus *Bacillus* and they were *Bacillus subtilis ,Bacillus thurengenesis, Bacillus stearothermophilus, Bacillus brevis, Bacillus mycoides* and *Bacillus megaterium* 2 (Table 4) (API). *Bacillus sp*. strains, identified with the API 50CH strips, fermented mostly Glycerol, L-arabinose, D-ribose, D- xylose, L-xylose, D-adonitol, D-galactose, Inositol, amygdalin, arbutin, salicin, D-maltose, D-lactose (bovine origin), D-saccharose (sucrose), D-trehalose, glycogen and xylitol. All *Bacillus* sp. were able to ferment Esculin Ferric Citrate.

Maximum bacterial load was found to be associated with the date variety Mabroon, followed by Khalas ,Barhi ,Mashrooq, Sukhari, Khodary, Ajwa , Naboot Sultan Amber, Naboot Seif , Rabeae and the least bacterial count was found the variety Segae. Mabroon showed the presence of *B. thuringenesis*. *B. subtilis* was isolated from all the fruit varieties but varying numbers except Sagae and Rabeae that showed its complete absence. However, it was high in the variety Barhi and Khalas.

The date fruit variety Barhi and Naboot Sultan showed the presence of all the six species of the genus *Bacillus* that is *B. subtilis, B. thurengenesis, B. stearothermophilus, B. brevis, B. mycoides,* and *B. megaterium. E. coli* was present in Khodary and Naboot Sultan only while Rabeae was the only variety which showed the presence of the contaminant *S. aureus.* Among all the date varieties screened for the presence of seed borne bacterial isolates, only two varieties that is Mabroon and Naboot Sultan showed bacterial contamination with *B. subtilis* and *B. brevis* while the rest of them did not yield any growth on the respective media (Table 5).

Table 3. Biochemical tests performed on Gram positive cocci and Gram negative rods.

Biochemical test	Result	Results
Gram positive cocci grape like sturcture	24	25
Catalase test	+	-
Coagulase test	+	-
Mannitol salt agar plate	yellow	-
Gram negative rods biochemical tests		
Methyl Red	-	+
Vogues Proskauer	-	-
Citrate	-	-
Urease	-	-
Eosine Methylene Blue plate	-	Green metallic sheen
MacConkey agar plate	-	Pink
Identification	*Staphyloccus aureus*	*Escherishia coli*

DISCUSSION

Moulds are considered to be the major causative agent of the spoilage of date fruits at all stages of ripening on trees as well as during storage and processing. Both seeds and fruits showed the presence of fungi in varying number. However, fruits were heavily contaminated than seeds. Fungi belonging to the Genus *Aspergillus* were present in high numbers in dry dates; the most predominant amongst them were the *A. niger, A. flavus A. fumigatus* and *A. nidulans.* Similar findings were reported by Ragab et al. (2001). Al Sheikh (2009) isolated 10 species of *Aspergillus,* of them *A. niger* predominated. Our results are also in accordance with Atia ((2011) who isolated *A. niger* as predominant fungi followed by *A. flavus.* A study conducted on date palm fruits collected from Figuig oasis of Morocco also showed A*. niger* to be the most prevalent of all the fungi isolated. During our study, some dry seeds and fruit of the cultivar Khalas and Sukhari were heavily contaminated with *A. niger* alone.

The predominance of black *Aspergilli* in dried fruits could be due to the protection provided by their black spores from sunlight and ultraviolet light thereby giving them a competitive advantage in this habitat. Moreover, high sugar concentration and low water activity in dried fruits also assist the development of these fungi because they are xerophilic in nature (Iamanaka et al*.,* 2005).

The second most dominant fungi in the fruits was *Alternaria alternate* followed by *Penicillium chrysogenum w*hereas the second most dominating seed borne fungi was *F. oxysporum* followed by *F. solani.* Presence of *Fusarium spp* in high numbers from seeds of different date varieties has been reported earlier (Al Sheikh, 2009; Bokhary, 2010). Additionally, *Fusarium spp* is known to cause Bayoud disease and has also been isolated from leaf rachis from different varieties and from different areas of central region of Saudi Arabia (Al-Shahwan et al., 1997; El-Hassni et al., 2007). *C. cladosporoides* was isolated in very low numbers in our study.

Other fungal genera like *Rhizopus stolonifer, Curvularia lunata*, *Chaetomium sp, Mucor sp* and other fungi were isolated with various frequencies and occurrence .Similar fungal species and many more were reported from different varieties of date fruits and seeds from markets and under different storage conditions from Saudi Arabia (Moore et al., 2011; Atia, 2011; Al Sheikh, 2009; Bokhary, 2010; Hamad, 2012). This high level of mold contamination can be attributed to the fact that dates are harvested in the dry windy months of July –September. Airborne mold spores can easily contaminate the fruits of tall palm trees (Hamad, 2012).

All the date fruit varieties screened, showed varying numbers of bacterial load.

Mabroon , Khalas and Barhi emerged as the most contaminated varieties while Naboot Sultan and and Barhi showed the presence of all the six species of *Bacillus* isolated during our screening; this could be attributed to moisture content, carbohydrates, nature of solutes, post harvest conditions like storage temperature, ripening stage or the cultivar itself could be considered as the major factors affecting the microbial load and as consequence the shelf life of dates (Rygg et al., 1956; Hasnaoui et al, 2010; Al Jasser,2010).

Isolation of some fungal species from all the samples of date fruit and seeds makes it evident that some molds are generally more tolerant to low water activity levels than bacteria and hence seems to be more persistent in the dry and hot conditions prevailing in the open markets whereas these condition are not conducive for the proliferation of endogenous bacterial flora which is clear from the low incidence of bacterial count from all the date

Table 4. Profiles of the biochemical tests using API 50CH/B Medium galleries for Identification of Bacillus strains.

API 50CH/B tests	Parameter	*B.thurengiensis*	*B.stearothermophilus*	*B.stearothermophilus*	*unidentified*	*B.stearothermophilus*	*B.subtilis*	*B.thurengiensis*	*B.brevis*	*B.subtilis*	*B.subtilis*	*B.mycoides/thurengiensis*	*B.brevis*	*B.subtilis*	*B.subtilis*	*B.subtilis*	*B.brevis*	*B.megaterium2*	*B.megaterium2*	*B.subtilis*	*B.brevis*	*B.thurengiensis/mycoides*	*B.thurengiensis*	*B.subtilis*
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
	0 Control	–	–	–		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Glycerol	–	+	+		–	+	–	–	+	+	–	–	+	+	+	+	–	–	–	+	–	–	+
	Erythritol	–	–	–		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	D-Arabinose	–	–	–		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	L-Arabinose	–	+	+		–	+	–	–	+	+	–	–	+	+	+	+	–	+	+	+	–	–	+
	D-Ribose	+	+	+		–	+	–	–	+	+	+	–	+	+	+	+	–	+	+	+	–	+	+
	D-Xylose	–	–	–		–	+	–	–	–	–	–	–	–	+	+	–	–	+	+	+	–	–	–
	L-Xylose	–	–	–		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	D-Adonitol	–	–	–		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Methyl-ß-D-Xylopyranoside	–	–	–		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	D-Galactose	–	–	–		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	D-Glucose	+	+	+		+	+	+	–	+	+	+	–	+	+	+	+	–	+	+	+	–	+	+
	D-Fructose	+	+	+		+	+	+	–	+	+	+	–	+	+	+	+	–	+	+	+	–	+	+
	D-Mannose	–	+	+		–	+	–	–	+	+	–	–	+	+	+	+	–	–	–	+	–	–	+
	L-Sorbose	–	–	–		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	L-Rhamnose	–	–	–		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Dulcitol	–	–	–		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Inositol	–	–	–		–	+	–	–	–	–	–	–	+	+	+	–	–	–	–	+	–	–	+
	D-Mannitol	–	+	+		–	+	–	–	+	+	–	–	+	+	+	+	–	+	+	+	–	–	+
	D-Sorbitol	–	+	+		–	+	–	–	+	+	–	–	+	+	+	+	–	+	+	+	–	–	+
	methyl-αD-Mannopyranoside	–	–	–		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	methyl-αD-Glucopyranoside	–	–	–		–	+	–	–	–	–	–	–	+	+	+	–	–	–	–	+	–	–	–
	N-AcetylGlucosamine	+	+	+		+	–	+	–	+	+	+	+	–	–	–	+	–	+	+	–	+	+	–
	Amygdalin	–	–	–		–	+	–	–	–	–	–	–	+	+	+	–	–	+	+	–	–	–	–
	Arbutin	–	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 4. Contd

API 50CH/B tests																							
D-Celiobiose	_	+	+	+	+	_	_	+	+	_	+	+	+	+	+	_	_	_	+	+	_	+	
D-Maltose	+	+	+	+	+	+	_	+	+	+	_	+	+	+	+	_	+	+	+	_	+	+	
D-Lactose (bovine origin)	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	+	–	–	–	
D-Melibiose	_	_	_	_	+	_	_	_	_	_	_	+	+	+	_	_	_	_	+	_	_	_	
D-Saccharose(sucrose)	+	+	+	+	+	+	_	+	+	+	_	+	+	+	+	_	+	+	+	_	+	+	
D-Trehalose	+	+	+	+	+	+	_	+	+	+	_	+	+	+	+	_	+	+	+	_	+	+	
Inulin	_	_	_	_	+	_	_	_	_	_	_	+	_	+	_	_	+	+	_	_	_	_	
D-Melezitose	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	+	+	_	_	_	_	
D-Rafinose	_	+	+	_	+	_	_	+	+	_	_	+	+	+	+	_	+	+	+	_	_	_	
Amidon(starch)	+	+	+	_	+	+	_	+	+	+	_	+	+	+	+	_	+	+	+	_	+	+	
Glycogen	+	+	+	+	+	+	_	+	+	+	_	+	+	+	+	_	+	+	+	_	+	+	
Xylitol	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	
Gentiobiose	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	
D-Turanose	_	_	_	_	_	_	+	_	_	_	_	_	_	_	_	+	_	_	_	_	_	_	
D-Xlyxose	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	
D-Tagatose	_	_	_	_	_	_	+	_	_	_	+	_	_	_	_	+	_	_	_	+	_	_	
D-Fucose	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	
L-Fucose	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	
D-Arabitol	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	
L-Arabitol	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	
Potassium Gluconate	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
Potassium 2-KetoGluconate	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
Potassium 5-KetoGluconate	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	

samples. Hasnaoui et al. (2010) reported in their study that samples with high sugar content (61-83%) and low water activity (aw=<0.90) showed very low incidence of endogenous bacteria indicating poor conditions for their proliferation. Most bacteria cannot grow when water activity is reduced below 0.90 (Leistner and Rodel., 1975; Mossel, 1975; Sawaya et al., 1989). The Nature of solutes besides the water activity influences the growth of micro-organisms and hence plays an important role (Horner and Agnostopoulous, 1973). *S. aureus* was absent from all the varities except Rabea while *E. coli* was isolated from two varieties; Khodary and Naboot Sultan .In a study conducted by Aidoo et al. (1996), the pre-packed dates purchased in Glasgow contained high levels of coliform, yeasts and moulds and little or very low levels of *S. aureus*. It is apparent that the mesophilic aerobic bacterial contaminants were quite sensitive to the conditions of low temperature storage, low water activity, high sugar content in the fruits and the low level of oxygen in the packages so that the majority of them could not survive. All samples were found contaminated with molds, but the amount of contamination varied from one cultivar to the other. It can be understood from our results that variation of microbial contaminants, from date fruit of one variety to another could be due to the chemical composition of cultivar which makes them more susceptible to attack during harvest or processing.

Additionally, the hawkers in local open markets

Table 5. Number of Bacterial Isolates (Count/plate/ml) identified from different varieties of date fruits and seeds.

Isolates from fruits	Date varieties screened											
	Ajwa	Amber	Barhi	Khalas	Khodary	Mabroon	Mashrooq	Sukhari	Segae	Naboot Sultan	Naboot Seif	Rabeae
B.thuringenesis	…	…	2	20	…	60	…	1	1	3	2	…
B.subtilis	20	11	50	50	15	10	10	20	…	5	2	…
B.stearothermo	…	…	10	9	5	13	10	…	…	5	7	2
B.brevis	…	…	5	…	…	15	…	…	6	2	…	…
B.mycoides	3	8	1	…	5	…	…	10	…	5	3	2
B.meagaterium2	1	1	1	2	…	…	20	4	1	2	2	…
Staphylococcus aureus	…	…	…	…	…	…	…	…	...	…	…	10
Escherishia coli	…	…	…	…	1	…	…	….	…	1	…	…
Isolated from seeds												
B. subtilis	…	…	…	…	…	5	…	…	…	3	…	…
B.brevis	…	…	…	…	…	15	…	…	…	1	…	…

do not store them at refrigeration temperatures, further creating conditions required for the microbes to proliferate.

Another important fact is that date fruits contain some antimicrobial components. For example, some varieties contain up to 2.5% tannins (Al Hooti et al., 1997; Myhara et al., 2000) which have been reported to cause growth inhibition to many species of fungi and bacteria (Nelson et al., 1997; Ishida et al., 2006).

Conclusion

The climatic conditions as affected by temperature and humidity during growth, ripening, harvest and storage determine the extent of microbial infestation of the date palm fruits. Additionally, the nature of solute and the physiochemical changes occurring during ripening make the fruits more susceptible to microbial attack which could be the reason for variation in the fungal and bacterial load of different varieties. However the molds being more tolerant to extreme of conditions in this microenvironment were found in high numbers when compared to bacteria.

A very high incidence of *A. niger* from all fruits and seeds is to be noted. It can be concluded that dates palm fruits sold as loose fruits especially in local open markets of Saudi Arabia harbor many fungi and bacteria.

This level contamination with microbes in fruits, often consumed without washing, is a matter of concern. Hence methods that will curb the microbial infestation of fruits at harvest, post harvest and at storage conditions are required and should be made mandatory.

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Efficacy of *Mannheimia haemolytica* A2, A7, and A2 and A7 combined expressing iron regulated outer membrane protein as a vaccine against intratracheal challenge exposure in sheep

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This experimental study was done on a total of 40 male lambs with the objectives of developing experimental vaccines from *Mannheimia haemolytica* serotypes A2 and A7 that express iron regulated outer membrane protein and *in vivo* evaluation of their efficacy. Lambs were categorized in to four experimental groups and vaccinated with 1 ml of vaccine containing 5 × 10^8 CFU/ml. Group 1 was vaccinated with *M. haemolytica* A2, group 2 with A7, group 3 with serotype A2 and A7 combination, and group 4 received saline as control. They were challenged intratracheally by the respective homologous serotype after 35 days of vaccination. Post challenge clinical investigation showed that significant higher rate of morbidity was seen in control group which was demonstrated by raised rectal temperature (by 0.5-1°C) and respiratory signs. From the total of 26 lambs challenged with live *M. haemolytica* A2 and A7, 6 (23.1%) and 4 (15.3%) lambs were found dead and sick, respectively. Higher mortality and morbidity were observed in unvaccinated control group; however, lesser was recorded in combined vaccinated group. Lung lesions of variable severity were observed in 13 (50.0%) lambs following challenge. From vaccinated groups, 5 (27.8%) lambs were found to have a +1 lung lesion score. All of the lambs in unvaccinated control group had scores between +2 and +3. There was a statistically significant difference ($p < 0.05$) between control and vaccinated groups, while no statistically significant difference ($p > 0.05$) was seen among vaccinated groups concerning lung lesion scores. Furthermore, the respective serotypes of *M. haemolytica* were successfully re-isolated from pneumonic lungs at a mean titre range of $10^{2.2}$ – $10^{8.1}$ CFU/g. In conclusion, lambs which received combined vaccine confer relatively good protective efficacy than *M. haemolytica* A2 or A7 vaccinated groups. Therefore, further study should be done on evaluation of antibody titer at different time points.

Key words: Challenge, efficacy, lambs, lung lesion score, *Mannheimia heamolytica*, morbidity, mortality.

INTRODUCTION

Pneumonia is a major cause of economic losses in sheep industry, and *Mannheimia haemolytica* is one of the infectious agents most frequently associated with pathologic damage of ovine respiratory tract that causes fibrinous and necrotizing lobar pneumonia and pleurapneumonia (Hazıroglu et al., 1994). *M. haemolytica* is an opportunestic pathogen that inhabits the nasopharynx and tonsils of cattle and sheep (Radostits et al., 2006) and is capable of

causing infection when the body's defense mechanisms are impaired (Haig, 2011). Environmental stress factors like inclement weather, shipment, weaning, overcrowding and complex interactions among several infectious agents can serve as cofactors for pathogenesis of pneumonic pasteurellosis (Kraabel and Miller, 1997; Ganheim et al., 2005).

A major problem in the control of pneumonic pasteurellosis is the lack of vaccine which consistently induces protective immunity against *M. haemolytica* (Dyer, 1982). A number of live and killed vaccines have been developed and used, but their efficacy in field trials has been variable, ranging from no effect to reduced or even increased morbidity and mortality (Catt et al., 1985; Chengappa et al., 1988; Confer et al., 1988).

The composition of the bacterial surface is an influential factor in the interactions between pathogens and host defences (Costerton, 1988). The availability of iron appears to modify the surface composition of many pathogens (Neilands, 1982; Brown and Williams, 1985; Ikeda and Hirsh, 1988) including *M. haemoytica* (Deneer and Potter, 1989; Ogunnariwo and Schryvers, 1990) and these surface alterations may have a consequence in the pathogenesis of the respective disease. A variety of antigens, which may serve as potential immunogens, have been isolated from *M. haemolytica*. Outer membrane proteins (OMPs) of *M. haemolytica* seemed to be most important for stimulating immunity (Mosier et al., 1989a) and could be used in vaccine preparations (Gatewood et al., 1994; Pati et al., 1996).

M. haemolytica A2 is the most common isolate from pneumonic lungs of sheep and goats throughout the world (Bahaman et al., 1991; Gilmour et al., 1991a; Davies and Donachie, 1996), and most research activities on the development of *Mannheimia* vaccine have focused on incorporating either a suitable isolate of *M. haemolytica* A2 or immunogenic antigens extracted from the serotype (Bahaman et al., 1991; Mosier, 1993). The outer membrane proteins of *M. haemolytica* A7 were effective in protecting animals against homologous and heterologous infection of live *M. haemolytica* A2, A7 and A9 (Sabri et al., 2000).

Despite annual vaccination programs against pasteurellosis using killed *P. multocida* biotype A containing vaccine (Ovine pasteurella vaccine), produced at the National Veterinary Institute, Ethiopia, high mortality and morbidity has been reported by farmers and veterinarians. Currently there is no effective vaccine that protects sheep from strains of *M. haemolytica* derived from different geographical origins. However, given the significant economic losses due to sheep pneumonia caused by this bacterium, it is necessary to develop a vaccine for the benefits of sheep industry. Therefore, the objectives of this experimental study are to develop *M. haemolytica* serotypes A2, A7, and A2 and A7 combined experimental vaccine that express iron regulated outer membrane protein and to conduc*t in vivo* evaluation of their efficacy by challenge protection, lung lesion score and bacterial count.

MATERIALS AND METHODS

Experiment Animals

This experimental study was conducted at National Veterinary Institute (NVI), Debre Zeit, Ethiopia. A total of 40 male conventionally reared lambs, aged 6 – 9 months, with no history of vaccination against pneumonic pasteurellosis and free from clinical signs of pneumonia were selected for this study. They were individually identified with ear tags and dewormed with albendazole (7.5 mg/kg body weight) upon arrival at the experimental station. The trial was started only when all lambs were kept for two weeks of adaptation period and were screened for *M. haemolytica* A2 and A7 by indirect haemagglutination test. Animals were randomly assigned into four experimental groups and they were kept in identical environmental and management facilities in the same paddock.

Experimental vaccines and adjuvant preparation

The experimental vaccine used in this study was prepared at NVI Bacteriology Laboratory, Ethiopia. *M. haemolytica* serotypes A2 and A7 were selected and used as candidate vaccine strains based on their higher rate of isolation from different parts of Ethiopia (Zeleke, 1998; Teferi, 2000; Sisay and Zerihun, 2003; Ayelet et al., 2004; Mulate, 2007). These strains were isolated and identified from different outbreaks of pneumonic pasteurellosis in the country based on sugar fermentation (arabinose and trehalose) and the organisms were serotyped based on capsular antigens using indirect haemagglutination test with *M. haemolytica* known serotype antisera and stored at NVI germ bank in a lyophilized formed at -20°C.

The lyophilized form of *M. haemolytica* was cultured in tryptose soy broth (TSB) enriched with 10% horse serum. The tubes were pre-incubated for 48 h to check its sterility and the tubes with no contamination were used for bacterial growth. Serotype A2 and A7 were inoculated into the media separately and incubated at 37°C for 18 h. Bacterial growth was checked by examination of smears, turbidity and pH of the media.

Bacterial growth suspension that was harvested from TSB was cultured on tryptose soy agar (TSA), sheep blood agar and MacConkey agar. Purity, colony characteristics, haemolytic effect, oxidase test and growth on MacConkey agar were used as primary identification procedure. Analytical profile index (API) 20NE were used for biochemical identification. Finally, the identity of the isolates was confirmed serologically using IHA.

Iron restricted growth was achieved by growing the bacteria in 100 ml brain heart infusion (BHI) broth supplemented with iron chelator (2,2'- Dipyridyl) at a concentration of 150 µM. The culture was incubated at 37°C with rotatory shaking at 80 oscillations per minute for 18 hr as described by Gilmour et al. (1991b) and Confer et al. (1995).

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Table 1. Summary of the experimental design for vaccination and challenge infection.

Group	Vaccine type	No. of lambs	Vaccine dose (ml)	Type of challenge and no. of lambs	Inoculum Volume (ml)
1	A2	10	1	Live *M. haemolytica* A2 (5 lambs)	4
2	A7	10	1	Live *M. haemolytica* A7 (5 lambs)	4
3	Bivalent (A2 and A7)	10	1	Live *M. haemolytica* A2 (4 lambs) and A7 (4 lambs)	4
4	Sterile saline water	10	1	Live *M. haemolytica* A2 (4 lambs) and A7(4 lambs)	4

The bacterial mass in the vaccine preparation was determined using titration and standard plate count method as described in Quinn et al. (2002). Counts were made on titrations that yield 30 to 300 colonies per plate. The viable count for each serotype used in the vaccine was 5 x 10^8 CFU/ml.

Inactivation of the culture was done using 0.5% formaldehyde and aluminum potassium sulfate (Alum) (10%) was used as an adjuvant.

Vaccination experimental design

At the start of the experiment, animals from group 1-3 were injected subcutaneously with 1 ml 5x10^8 CFU/ml of *M. haemolytica,* grown under iron restriction, and formalin-killed, with aluminum potassium sulfate adjuvant. Group 1 received the vaccine containing *M. haemolytica* serotype A2; group 2 received the vaccine containing *M. haemolytica* serotype A7; and group 3 received *M. haemolytica* serotype A2 and A7 combined vaccine. Group 4 was injected in the same manner using sterile saline water and used as unvaccinated controls (Table 1).

Challenge infection

Animals were immunosupressed using dexamethason at a dose of 0.04 mg/kg body weight for three consecutive days (Charley, 1990) before challenge. According to Sabri et al. (2000), five weeks after vaccination, 5 animals from group 1 were challenged intra-tracheal with 4 ml inoculum containing 5.2 x 10^9 CFU/ml of live *M. haemolytica* A2. Similarly, 5 animals from group 2 were challenged intra tracheal with 4 ml inoculum containing 5 x 10^9 CFU/ml of live *M. haemolytica* serotype A7. Group 3 was split into two groups: each containing 5 animals and one of the group was challenged with A2 and the other group by A7. Finally, half of the control group (5 animals each) was challenged with A2 and the other half with A7 as shown in Table 1.

Following the intratracheal challenge, all lambs were observed daily for signs of respiratory infection and death before the surviving lambs were slaughtered on day 10 post-challenge. Lambs were considered febrile when the mean rectal temperature was >39.1 according to Robertshaw (2004). The entire respiratory tracts were examined and the extent of the lung lesions was determined according to the method described by Akan et al. (2006).

Vaccine efficacy evaluation

The efficacy of the vaccine was determined based on the resistance of vaccinated lambs against *M. haemolytica* infection, or severity of pneumonia post challenge, the lung lesion score and dose of the bacterial titer from lung lesions.

Clinical investigation and necropsy following challenge

Rectal temperatures were recorded twice a day for 10 consecutive days and lambs were observed closely for clinical symptoms mainly related to respiratory signs including death if present. Necropsy was performed following 10 days post challenge and lungs were examined for *M. haemolytica*-induced lesions and scored accordingly. The time of death was used to categorize the infection as peracute (< 48 hrs), acute (3-7 days), sub acute (> 8 days) according to Shafarin (2009). Based on the percentage of consolidated lung masses, lung lesions were scored as follows: 0 (lungs without lesions), +1 (1 – 4% consolidation), +2 (5 – 14% consolidation), and +3 (≥15% consolidation) according to Akan et al. (2006).

Bacterial isolations and colony count

Samples like heart, blood, lung, liver, thoracic fluid and mediastinal lymph nodes were collected immediately after slaughter. The specimens were processed for *M. haemolytica* re-isolation. Suspected colonies were identified as *M. haemolytica* by Gram staining and biochemical tests. Samples from which *M. haemolytica* could not be isolated were re-cultured for a maximum of three times before they were considered negative and discarded.

From each sample, serial ten-fold dilution in sterile BHI broth was made, and 0.1 ml volume of appropriate dilution were inoculated on two plates (blood agar plate and tryptose soy agar plate) per dilution. After overnight incubation at 37°C, plates that yield between 30 and 300 colonies were counted and the average of the two plates were taken. Plates that show absence of growth was re-incubated for 24 h. Representative colonies were serotyped based on capsular antigens using IHA test with *M. haemolytica* serotype antisera as described by Quinn et al. (2002).

Statistical analysis

Data generated from this experiment was recorded and stored in MS Excel 2007. Results were statistically analyzed using SPSS software versions 19.0 for MS windows. Descriptive statistics were used to analyze the clinical parameters post challenge. Pair wise comparison using Wilcoxon rank test was used for comparing lung lesion score among the different control and vaccinated groups.

RESULTS

Prior to vaccination, all experimental lambs were screened for the presence of antibody against *M. haemolytica* serotypes A2 and A7 using indirect haemagglutination test and all were found negative. All

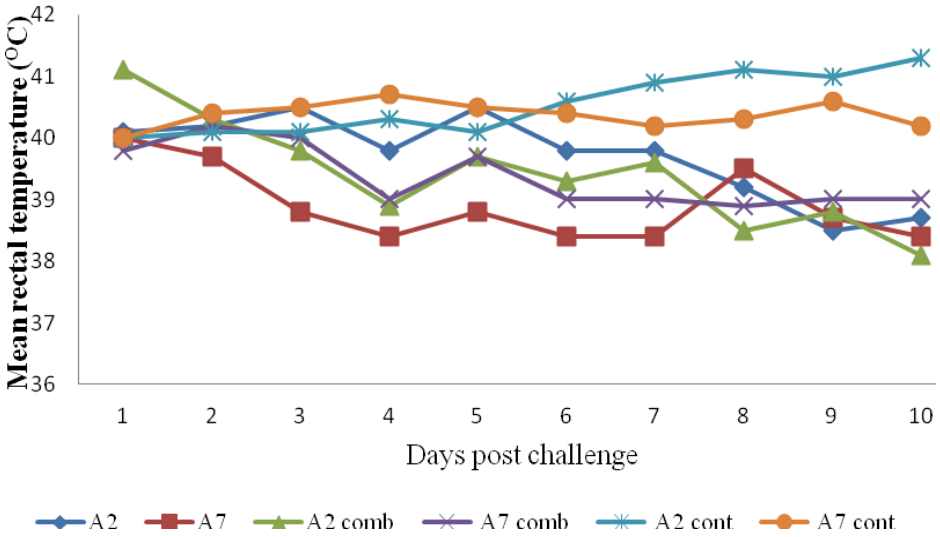

Figure 1. Mean rectal temperature for ten consecutive days post challenge.

Table 2. Summary of animals vaccinated with *M. haemolytica* serotypes, challenge serotypes, number of dead and sick animals, and number of lambs with lung lesions.

Vaccine used	Challenge serotype	No, of lambs challenged	No. of dead lambs	No. of sick lambs	No. of lambs with lung lesions	Mean counts of. *M. haemolytica* in lungs (log_{10} per g)
M. haemolytica A2	A2	5	1(20%)	1(20%)	2(40)	$10^{3.5}$
M. haemolytica A7	A7	5	1(20%)	0	1(20%)	$10^{3.1}$
M. haemolytica A2-A7 combination	A2	4	0	0	1(25%)	$10^{3.8}$
	A7	4	1(25%)	0	1(25%)	$10^{2.2}$
Unvaccinated control	A2	4	1(25%)	2(50%)	4(100%)	$10^{8.1}$
	A7	4	2(50%)	1(25%)	4(100%)	$10^{7.6}$
Total		26	6(23.1%)	4(15.3%)	13(50.0%)	

lambs were daily monitored and clinical signs were recorded following challenge. The mean rectal temperature was raised from 0.5 – 1°C starting from day 1 post challenge in all vaccinated groups up to day 3 post challenge, but the rectal temperature remained raised for 10 days post challenge in the control group as shown in Figure 1.

The prominent clinical signs revealed in very sick lambs were high rectal temperatures (≥ 40°C) and tachypnoea or dyspnoea and death of the animals within 48 h post challenge. Mildly affected lambs showed anorexia, coughing, mucoid nasal discharge and dullness. All unvaccinated controls had signs of respiratory tract infection of variable severity and raised rectal body temperature throughout the period of observation following challenge.

From the total of 26 lambs challenged with live *M. haemolytica* serotypes A2 and A7, 6 (23.1%) and 4 (15.3%) lambs were found dead and sick, respectively. In this study, in unvaccinated control group, death was seen within 48 h post challenge, while deaths from vaccinated groups were found within 3-7 days post challenge. No deaths of lambs were observed after 8 days post challenge. Higher mortality and morbidity were observed in the unvaccinated control group and lesser were recorded in lambs which received A2 and A7 *M. haemolytica* combined vaccine as illustrated in Table 2. From the total of 26 lambs challenged with live *M. haemolytica* A2 and A7, 13 (50.0%) lambs had lung lesions as shown in Table 2.

Lung lesions score

M. haemolytica serotype A2 vaccinated group had two (40%) lambs with +1 lung lesions score as shown in Figure 2, while *M. haemolytica* serotype A7 had one

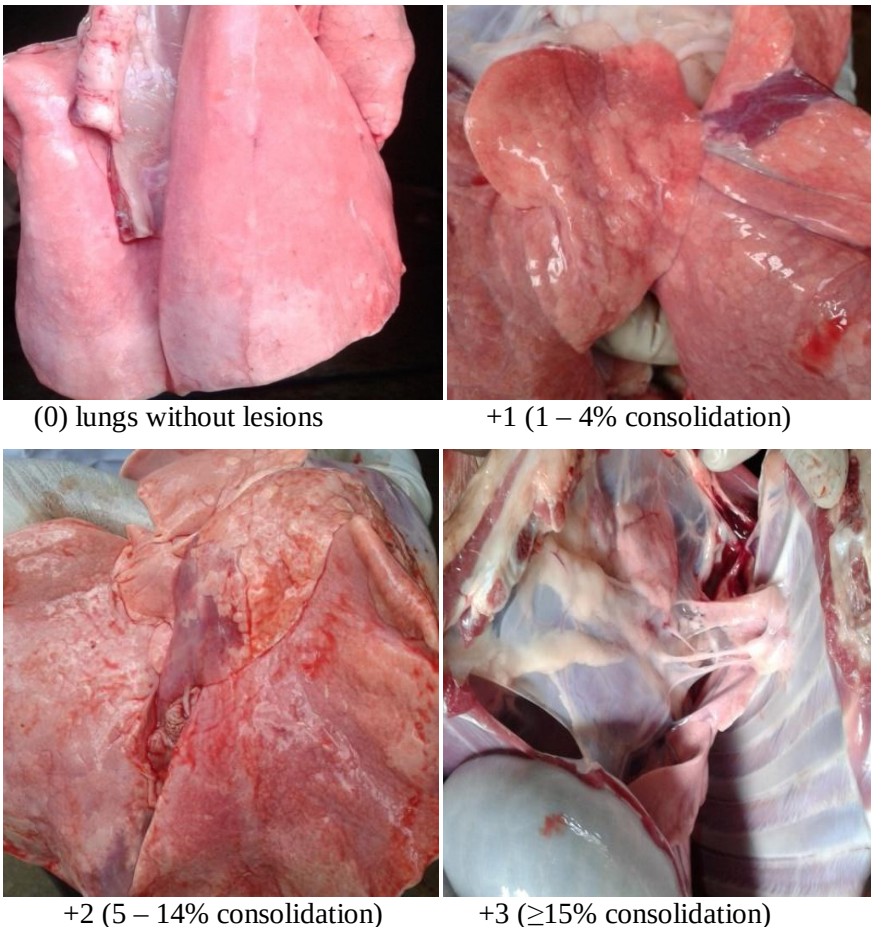

Figure 2. Lung lesions score observed after necropsy.

(20%) lamb. When the necropsy findings were evaluated comparatively, there was a statistically significant difference ($p < 0.05$) between the control and the vaccinated groups with regards to lung lesion scores, while there was no statistically significant difference ($p > 0.05$) among *M. haemolytica* A2, *M. haemolytica* A7, and A2-A7 *M. haemolytica* combined vaccinated groups.

Severe lung lesions ranging from +2 to +3 were observed in all 8 (100%) unvaccinated control group challenged by live *M. haemolytica* serotype A2 and A7 while 5 (27.8%) lambs were found as +1 lung lesion score from vaccinated groups. Statistically significant difference ($p < 0.05$) in lung lesion scores was observed between the vaccinated and unvaccinated control group as shown in Table 3. A strong statistically significant difference ($p = 0.015$) was observed between A2 and A7 *M. haemolytica* combined vaccinated and the unvaccinated control group. However, there was no statistically significant difference ($p > 0.05$) among vaccinated groups. The less extensive lung lesions was found in *M. haemolytica* A2 and A7 combined vaccinated groups when compared with separate serotype A2 and A7 vaccinated groups.

Microbiological isolations

Tissue and fluid samples including heart blood, lung, liver, thoracic fluid and mediastinal lymph nodes were collected from each lamb immediately after necropsy and were processed for re-isolation of *M. haemolytica*. The respective serotypes of *M. haemolytica* were successfully re-isolated from all lungs showing pneumonic lesions, but none of the lungs without pneumonic lesions yielded *M. haemolytica*. The organisms were re-isolated from all pneumonic lungs in both vaccinated and unvaccinated control groups, but were isolated from all tissue specimens in unvaccinated control group. *M. haemolytica* were isolated from the lung lesions at a mean titre range of $10^{2.2}$ - $10^{8.1}$ CFU/g as shown in Table 2.

DISCUSSION

In this study, an experimental vaccine efficacy against *M. haemolytica* infection in lambs was investigated based on development of pneumonia, lung lesion score and bacteriological findings following challenge. The study

Table 3. Pair wise comparisons of lung lesions score for vaccinated and control groups.

Group	Compared groups	Standard deviation	Mean rank		Sum of rank		P-value
			Positive rank	Negative rank	Positive rank	Negative rank	
A2	A7	0.548	0.00	1.00	0.00	1.00	0.317
	A2 and A7 combined	0.447	2.00	2.00	2.00	4.00	0.564
	Control	0.535	3.00	0.00	15.0	0.00	0.041
A7	A2	0.548	1.00	0.00	1.00	0.00	0.317
	A2 and A7 combined	0.447	1.50	1.50	1.50	1.50	1.000
	Control	0.535	3.00	0.00	15.0	0.00	0.038
A2 and A7combined	A2	0.447	4.00	2.00	4.00	2.00	0.564
	A7	0.548	1.50	1.50	1.50	1.50	1.000
	Control	0.535	0.00	4.00	0.00	28.0	0.015
Control	A2	0.535	0.00	3.00	0.00	15.0	0.041
	A7	0.548	0.00	3.00	0.00	15.0	0.038
	A2 and A7 combined	0.447	0.00	4.00	0.00	28.0	0.015

was performed by using experimental *M. haemolytica* serotype A2, A7, and A2-A7 combined vaccine, grown under iron restriction, in four trial groups of lambs followed by challenge with homologous serotype of *M. haemolytica*. The *Mannheimia* serotypes contained in this experimental vaccine appear to be appropriate for Ethiopian conditions, because they have been shown to occur at a high frequency in different parts of the country (Zeleke, 1998; Teferi, 2000; Sisay and Zerihun, 2003; Ayelet et al., 2004; Abera, 2005; Mulate, 2007) and did not have a vaccine produced in Ethiopia against these serotype. The most important result of this study was that vaccination with A2 and A7 *M. haemolytica* combined vaccine confer relatively good protective efficacy than *M. haemolytica* A7 or A2 vaccinated groups.

Lambs were monitored daily, from days 0 through 10, post challenge for the presence of clinical signs related to respiratory problems. The mean rectal temperature was found raised by 0.5 – 1°C for 3 days post challenge in all the unvaccinated control and vaccinated groups. This is in agreement with the study of Akan et al. (2006) who demonstrated that rectal temperature had been elevated up to 1°C in the treatment groups for the first three days post challenge. The rectal temperature declined after three days of post challenge in the vaccinated groups, but failed to reduce in the control group in this study. Mekonnen (2012) reported a 1.5°C rise of rectal temperature in the control group than all vaccinated lambs with capsular antigen of *M. haemolytica.*

The clinical signs were evaluated and revealed that very sick lambs had high rectal temperatures (≥ 40°C) and tachypnoea or dyspnoea and died within 48 h post challenge; mildly affected lambs showed signs like anorexia, coughing, oculo-nasal discharge and dullness, and survived until necropsy. Less severe clinical signs related to respiratory problems and fewer deaths were recorded in vaccinated groups than unvaccinated control group. This result is in agreement with the work of Odugbo et al. (2006) who reported that the prominent clinical signs were anorexia, mucoid nasal discharge, coughing for lambs infected with *P. multocida*. This variable clinical signs might be due to the protective effect of the vaccine against *M. haemolytica* infection.

From the total of 26 lambs challenged with live *M. haemolytica* serotype A2 and A7, 6 (23.1%) and 4 (15.3%) lambs were found dead and sick, respectively. In unvaccinated control group, death was seen within 48 h post challenge, while deaths were found within 3-7days post challenge from vaccinated groups. High mortality was observed in the unvaccinated control group than vaccinated groups. This result demonstrated that unvaccinated lambs have less protection against *M. haemolytica* infection. That might be due to lack of protective antibody against *M. haemolytica* serotypes.

When the necropsy findings were evaluated comparatively, there was a statistically significant difference ($p < 0.05$) between control and vaccinated groups concerning lung lesion scores, while there was no statistically significant difference ($p > 0.05$) among vaccinated groups. Severe lung lesions ranging from +2 to +3 were observed in all unvaccinated control group challenged with live *M. haemolytica* serotypes A2 and A7. Significant decrease in lung lesions after challenge with *M. haemolytica* strains following vaccination showed that protection using iron regulated outer membrane protein showed that vaccine of *M. haemolytica* strains is an effective method against an infection with homologous strains; however, no comments was made on cross-protection with heterologous strains because challenging was not performed with other serotypes in this study. A strong statistically significant difference ($p = 0.015$) were observed between *M. haemolytica* A2 and A7 combined

vaccinated group and unvaccinated control group. This indicated that less extensive lung lesions were found relatively in *M. haemolytica* A2 and A7 combined vaccinated group as compared to separate serotype A2 and A7 *M. haemolytica* vaccinated groups. This finding was similar to the work of Mekonnen (2012) who reported that vaccine prepared from the capsular antigen of combined *M. haemolytica* A2 and A7 strains provide significantly less extensive lung lesion score than the control, *M. haemolytica* A2, and A7 vaccinated groups.

The respective serotypes of *M. haemolytica* were successfully re-isolated from all pneumonic lung lesions. *M. heamolytica* was re-isolated from all samples (heart, blood, lung, liver, thoracic fluid, and mediastinal lymph nodes) in unvaccinated control groups, but they were re-isolated only from the lung tissue sample in vaccinated groups. This failing of isolation of this organism from other tissues might be due to the protective effect of the vaccine evidenced by limited bacterial population. This finding is supported by the work of Quinn et al. (2002).

High mean bacterial titer was found in unvaccinated control group challenged by live *M. haemolytica* A2 ($10^{8.1}$ CFU/g) and *M. haemolytica* A7 ($10^{7.6}$ CFU/g) where as the least was recorded in *M. haemolytica* A2 and A7 combined vaccinated group ($10^{2.2}$ CFU/g). This high bacterial titer has demonstrated that the unvaccinated control group has failed to protect against challenge. Gilmour (1980) revealed that in sub-acute cases which have not been treated with antibiotics *M. haemolytica* counts in excess of $\log10^{7}$ CFU/g of lung lesion are usually obtained.

In conclusion, vaccine containing iron regulated outer membrane protein expressed *M. haemolytica* A2, A7, and A2 and A7 combined vaccine significantly provide protection against homologous strains.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Differential immunoreactivity of the root-knot nematodes, *Meloidogyne graminicola* and *Meloidogyne incognita* to polyclonal and monoclonal antibodies and identification of antigens through proteomics approach

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Meloidogyne graminicola **infect graminaceous plants but have lesser tendency to infect dicotyledonous plants.** ***Meloidogyne incognita*** **is a pest of dicots and occasionally infects cereals. Evolutionary adaptation of these root-knot nematodes to their preferred hosts might have led to variability in their gene/protein profile which could contribute to their differential behaviour outside and inside the different host crops. Polyclonal and monoclonal antibodies raised against several nematode species showed cross-reactivity to antigens with different molecular weights present in the whole body homogenate of** ***M. incognita*** **and** ***M. graminicola*** **J2. This variability in antigenicity may correspond to specific functions of these molecules in** ***M. incognita*** **and** ***M. graminicola*****. Using proteomics approach possible amino acid sequence of those antigens was elucidated and showed sequence similarity with several proteins like signal recognition particle protein, galactose binding lectin, zinc finger motif, neurotransmitter gated ion channel, transmembrane protein, etc. from the genomic database of several nematode species. To investigate the function of the identified nematode genes, RNA interference could be used to reduce the expression of these selected genes and determine their importance for nematode development, survival or parasitism.**

Key words: Antibodies, antigens, host recognition, secreted-excreted products, surface coat.

INTRODUCTION

Plant parasitic nematodes (PPN) are one of the major limiting factors in crop production worldwide damaging up to 10% of world's agricultural output equivalent to $157 billion annual monetary loss (Abad, 2008). PPNs are unique in their ubiquitous nature and persistence in the soil. The conflicting nature of their attack allows their presence to often pass unnoticed while crops slowly decline in vigour and yield. Rarely is any crop free from attack of these tiny and microscopic pathogens.

The molecular dialogue between PPNs and the host starts at a distance, with modifications of the surface of Infective Juveniles (J2) in response to root diffusates.

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During co-evolution with the host plant, parasitic nematodes have developed the capacity to recognize and respond to chemical signals of host origin. Signals from roots present in the rhizosphere and bulk soil can specifically influence nematode behaviour, inducing hatching, attract-tion, surface cuticle changes, root exploratory behaviour and penetration of plant roots, and involve molecular communication between the nematode and respective host plant (Curtis et al., 2011).

Once the nematode has reached the root, different secretory organs participate in the molecular interaction with the host. Feeding cell formation is presumably initiated in response to signal molecules released by the parasitic J2, but the nature of the primary stimulus is unknown, as is the host target for the presumed nematode ligand(s), which must be transduced to elicit the feeding site. The most widely held hypothesis is that the necessary metabolic re-programming of root cells is triggered by specific nematode secretions, which presumably interact with membrane or cytoplasmic receptors in the plant to switch on cascades of gene expression that alter cell development. Secretions from the cuticle build up a surface coat (SC) that is likely to hide the nematode from host perception throughout the interaction (Curtis, 2011).

The nematodes possess an elaborate nervous system which plays a great role in recognition of host roots, appropriate feeding or penetrating sites, selection of tissue for migration in root and feeding cell/site formation. Amphids are the primary structures for chemoreception of the chemical cues which orient nematodes towards food sources and lead to infection. Disruption of the sensory functions involved in nematode interactions with plants, that is, disruption of host recognition process is one of the innovative management tactics (Spence et al., 2008).

Rapid advances in protein analytical technologies, makes mass spectrometry-based interactive proteomics a method of choice for analyzing functional protein com-plexes. A combination of 2D-gel electrophoresis with micro-sequencing has led to the identification of two endoglucanases and a novel protein in the secretions of the cyst nematode, *Heterodera schachtii* (De Meuter et al., 2001).

A calreticulin and a 14-3-3 protein identified in the secretion of *M. incognita* had multiple functions including regulation of cell signaling and metabolic pathways along with the control of the cell cycle (Jaubert et al., 2002; Abad et al., 2003). An annexin gene (*Gp-nex-1*) and putative collagen gene (*gp-col-8*) were isolated from a *Globodera pallida* expression library by screening with a polyclonal and a monoclonal antibody (MAb), respect-tively, both antibodies reacted with antigens present in the amphids of *Globodera* sp. (Jones et al., 1996; Gray et al., 2001; Fioretti et al., 2001). MAb directed against amphidial secretions interfered with nematode invasion of plants and therefore secretions from the amphids might be involved in host-recognition processes (Fioretti et al., 2002).

Root-knot nematodes (*Meloidogyne* spp.) are one of the most damaging agricultural pests, attack almost every crops. A large number of host range studies have shown that some species like *Meloidogyne incognita, Meloidogyne javanica, Meloidogyne arenaria, Meloidogyne hapla* etc. characteristically prefer dicotyledonous crops and rarely infect cereals. On the contrary, another group including *Meloidogyne graminicola, Meloidogyne naasi, Meloidogyne oryzae, Meloidogyne salasi, Meloidogyne triticoryzae* etc. generally prefer cereal hosts but can also infect some dicotyledonous plants (Dutta, 2012). Preliminary attraction bioassay studies in our laboratory have shown that root-knot nematodes (*M. incognita* and *M. graminicola*) are attracted differently to good hosts and poor hosts whilst no attraction was observed for non-host plants (Reynolds et al., 2011; Dutta et al., 2011). Understanding the complexity of molecular signal exchange and response during the early stages of the plant-nematode interactions is important to identify vulnerable points in the parasitic life cycle that can be targeted to disrupt nematode host recognition. Thus, attempts were made to identify the proteins related to host recognition process of root-knot nematodes, *M. incognita* and *M. graminicola* through proteomics approach.

MATERIALS AND METHODS

Culturing of nematodes

M. incognita (Kofoid & White) Chitwood and *M. graminicola* Golden & Birchfield were maintained respectively, on tomato (*Solanum lycopersicum* cv. Tiny Tim) and rice (*Oryza sativa* cv. Ballila) in a glasshouse. Egg masses were collected on a piece of 10 µ porous cloth supported on Miracloth (Calbiochem, U.K.) held by two plastic rings in a flat bottomed evaporating dish containing distilled water (Hooper, 1986). Freshly hatched second stage juveniles (J2) were used for all the experiments.

Antigen preparation

A pellet of several thousand J2 of each nematode species were homogenized in 0.01 mM PBS pH 7, on ice using a homogenizer (Biomedix) from which whole body homogenates were obtained. Cuticle surface antigens were collected with 1% Triton X-100, Tris 0.125 M, pH 7.5. Stylet secretions were induced by adding 0.2 mg/ml of 5 methoxy-*N, N* dimethyl tryptamine (Sigma) which enhances stylet thrusting (Goverse et al., 1994; Curtis, 1996) (Figure 1). Proteins were quantified with Biorad protein assay.

Antibodies

The polyclonal antibodies (PAbs) were raised in rabbits to several plant parasitic nematodes: IACR-PC 373 (homogenates of *M. incognita* J2), IACR-PC 374 (live pre-parasitic J2 of *M. incognita*), IACR-PC 353 (live pre-parasitic J2 of *Heterodera avenae*), IACR-PC 389 (SC extract of *M. incognita*), IACR-PC 418 (SC extract of *M. arenaria* race from Portugal) and IACR-PC 419 (SC extract of *M. arenaria*). Monoclonal antibody (MAbs) IACR-CCNj.2a.15 raised in mouse immunized 3 times intraperitoneally with whole J2 and secreted-excreted (SE) products of the cereal cyst nematode *H. avenae* (Curtis, 1996) was used in this study.

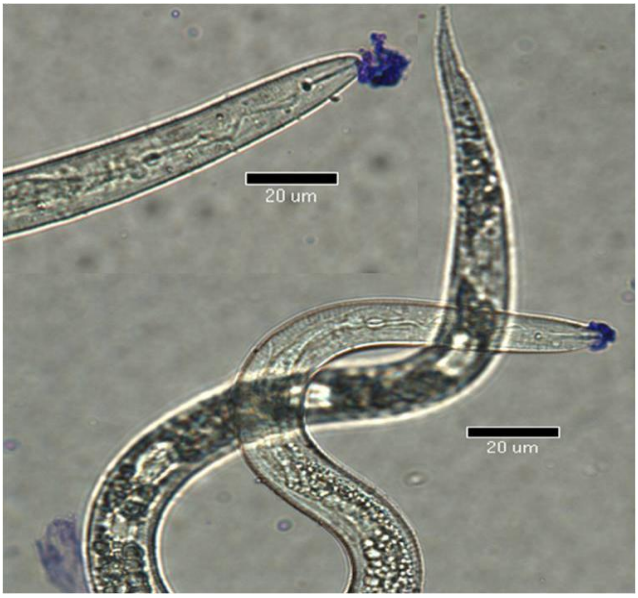

Figure 1. Induction of stylet secretion in *M. graminicola* J2. Nematodes treated with the neurotransmitter showed massive amounts of stylet secreted proteins around the stylet tip at 4 h of incubation. Coomassie brilliant blue R250 was added to the suspension to visualize secreted proteins.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

One dimensional SDS-PAGE using a 15% (w/v) acrylamide in the separating gel and 4% (w/v) acrylamide in the stacking gel were performed (Laemmli, 1970). Sample buffer (10% glycerol, 5% 2-mercaptoethanol, 2.3% SDS and 0.01% bromophenol blue in 0.5 M Tris HCl pH 6.8) was added to the protein samples (40 µg protein per lane). The proteins were separated using a vertical polyacrylamide slab electrophoresis tank followed by fixed and stained with Coomassie Blue G250 (Sigma-Aldrich Chimie) or silver stained or transblotted onto 0.2 µm nitro-cellulose (NC) membrane. The molecular weight markers (Pharmacia) used was: lactalbumin 14 kDa; soybean trypsin inhibitor 20 kDa; carbonic anhydrase 30 kDa; ovalbumin 43 kDa; bovine serum albumin 67 kDa and Phosphorylase b 94 kDa. The experiment was repeated at least thrice.

Western blotting

A Multiphor II Nova Blotting Electrophoresis Transfer Unit (Pharmacia) was used to transfer proteins from the gel to NC membrane in transfer buffer (39 mM glycine, 48 mM Tris, 0.0375% SDS, 20% methanol). The immunolabelling was performed after blocking the NC membrane overnight at 4°C in a solution of PBS, 0.1% Triton X-100, 5% Marvel dried milk (PBSTM). The NC membranes were incubated with the primary PAbs (IACR-PC 373 1:5000; IACR-PC 374 1:5000; IACR-PC 353 1:2000; IACR-PC 389 1:2000; IACR-PC 418 1: 2000; IACR-PC 419 1:2000) for 1 h at room temperature under agitation. The membranes were then washed in PBST and incubated with horseradish peroxidase conjugated anti-rabbit secondary antibody (diluted 1: 2000 in PBS) for 45 min on a shaker in dark. After further washes in PBST the membranes were treated with 10 ml of PBS containing 0.05% w/v diaminobenzidine and 30% H_2O_2 until bands were suitably dark (Harlow and Lane, 1988). Negative controls were non-immune serum and secondary antibody. Membrane was incubated with MAb for 2 h, washed and treated with peroxidase-conjugated goat anti-mouse polyvalent immunoglobulins (diluted 1: 1000 in PBS) for 45 min. Immunodetection was enhanced by chemiluminescence reagent (ECL, Amersham International plc). The blot was immersed in it for 1 min and exposed to Hyperfilm-ECL for 1 min to develop the film. Negative controls consisted of blots probed with tissue culture supernatant (20D medium) and an irrelevant monoclonal antibody. Three biological and three technical replicates were taken for each of the samples.

Protein sequencing

The major protein spots for both the nematode species were excised from Coomassie Brilliant Blue G250 stained gels. Gel pieces were destained, reduced and alkylated, digested with trypsin (Promega, UK) and obtained peptides were sequenced through MALDI-MS and ESI-MS (Lovegrove et al., 2009) in MASCOT server. MS raw data were acquired on the Data Directed Analysis feature in the MassLynx (Micromass) software with a 1, 2, 4 duty cycle (1 s in MS mode, two peptides selected for fragmentation, maximum of 4 s in MS/MS acquisition mode). MS/MS raw data were transferred from the QTOF Micro computer to a server and automatically manipulated for generation of peak lists by employing Distiller version 2.3.2.0 (http://www.matrixscience.com/distiller.html) with peak picking parameters set at 5 for Signal-Noise Ratio (SNR) and at 0.4 for correlation threshold (CT). Peak listed data were searched by employing Mascot (http://www.matrixscience.com) version 2.3.01 against the list of protein sequences predicted for *M. incognita* and *M. graminicola* using BLASTP server for their sequence similarity to known proteins of other nematode species at the NCBI database (Altschul et al., 1990).

Comparison with homologous sequences was done with ClustalW (Larkin et al., 2007). Theoretical isoelectric point (pI) and molecular weight (mw) for the conceptually translated protein sequences were calculated by the Expasy Protparam tool available at http://expasy.org/. Gene ontology term was assigned through AmiGO BLAST. Signal peptides were predicted by the SignalP server (Petersen, 2011). Secondary structure of the protein was predicted with an *ab initio* protein modelling server I-TASSER (Roy et al., 2010). This server uses the threading technique to predict the 3D models. The server generated 5 best models based on multiple-threading alignments and iterative template fragment assembly simulations along with their confidence scores. The 5 models were visualized by the Visual Molecular Dynamics (VMD) software models, different validation techniques were used. In a similar fashion, PROCHECK (Laskowski, 1996) and VERIFY 3D (Eisenberg, 1997) were used to validate the predicted protein structures. The PROCHECK software generates ramachandran plot which nicely explains the stereochemical configuration of amino acid residues. The VERIFY 3D analyses the compatibility of an atomic model with its amino acid sequence. Finally, the better model was adopted based on the aforementioned tools.

RESULTS AND DISCUSSION

SDS-PAGE

All silver staining methods rely on the reduction of ionic to metallic silver to provide metallic silver images, the selective reduction at gel sites occupied by proteins as compared to non-protein sites being dependent on differences in the oxidation-reduction potentials of these

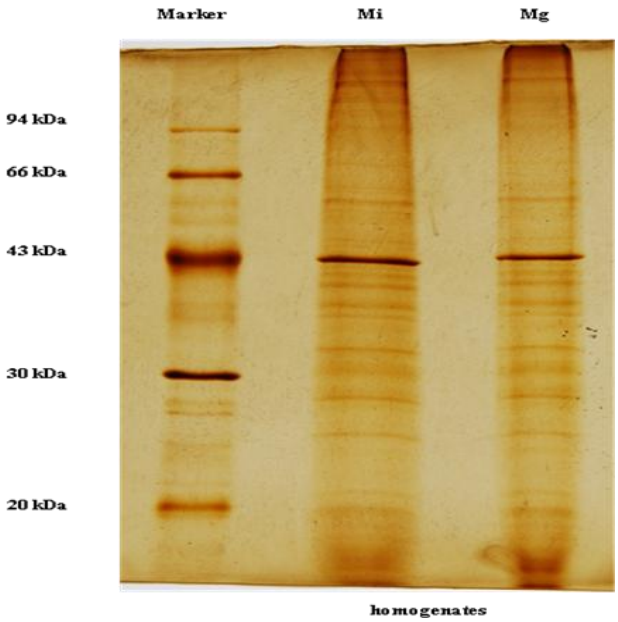

Figure 2. Analysis of homogenate proteins of *M. incognita* (Mi) and *M. graminicola* (Mg) by SDS-PAGE. Protein bands were visualized by silver staining.

sites; while Coomassie Blue binds strongly to arginine and lysine residues and with lower affinity to aromatic side chains (Simpson, 2003). Thus SDS-PAGE followed by silver staining was not enough to detect the differences in the secretion and homogenate proteins of both nematode species (Figure 2). But SDS-PAGE followed by Coomassie Blue staining did detect several polymorphic homogenate antigens among the two species which might have some role in the host recognition and parasitic life cycle of that two species inside different hosts (Figure 4).

Western blots

PAb raised against SC extract and SE products of *Meloidogyne* spp. showed antigenic cross-reactivity with the different molecular weight antigens of *M. incognita* and *M. graminicola* stylet exudates. Antigens from *M. incognita* and *M. graminicola* proteins shared some proteic epitopes and also reacted with the antibody at different bands when the blot was probed with the PAb raised against live pre-parasitic J2 of *M. incognita* (Figure 3a and b). It can be speculated that some of the antigens of *M. incognita* and *M. graminicola* recognized by several antibodies are continuously shed from the nematode SC and may also originate from SE products of nematodes. The origins of surface-associated antigens on nematodes may differ for various antigens. These non-structural proteins originate from gland cells such as excretory cells, pharyngeal glands, amphids and phasmids as well as from the hypodermis and rectal glands (Blaxter and Robertson, 1998; Hu et al., 2000). Glycosylated peptides have been reported to be present in abundance on the SC and SE products of several parasitic nematodes (Robertson et al., 1989; Schallig et al., 1994). These antigens may participate in the infection process by binding to proteins/receptors on the plant cell plasma membrane or modulate changes via signal transduction. Little is known about the roles of the surface antigens of plant-parasitic nematodes in pathogenicity. A more dynamic role as an elicitor in the determinative phase of nematode-plant interaction has been postulated for these surface molecules (Kaplan and Davis, 1987). Cuticular exudations appear to correlate with feeding periods of the nematode, implying that they might play a more sophisticated role in the infection process (Endo, 1993). Several proteins from the cuticle and amphids have previously been identified using antibodies (Atkinson et al., 1988; Davis et al., 1992; Stewart et al., 1993; Curtis, 1996; De Boer et al., 1996a, b). A putative role has been suggested for an amphid-secreted protein, which might be involved in the early steps of recognition between (resistant) plants and (avirulent) nematodes (Semblat et al., 2001).

PAb raised against whole body homogenates of *M. incognita* showed very strong reaction with the antigens of both *M. incognita* and *M. graminicola* in several similar and different band positions (Figure 3c). While, MAb raised against whole J2 and SE products of *H. avenae* showed very high level of cross reactivity with a couple of specific antigens of *M. incognita* proteins but surprisingly did not show any reaction with the *M. graminicola* stylet secreted proteins (Figure 3d). This might indicate that the common antigens (sharing proteic epitopes) perform the same function in the larval stages of *M. incognita* and *M. graminicola* during host recognition, invasion and development inside the cereal and dicotyledonous crops. While antigenic differences (recognized by antibodies at different molecular weight) among the two nematode species might account for adaptations of the proteins to allow parasitism in different hosts. This variability in antigenicity may also correspond to specific functions of these antigenic molecules in *M. incognita* and *M. graminicola*. Variability in antigenic properties of isoforms might be important for the survival of the parasite in the host (Overath et al., 1994).

Protein sequencing

Attempts were made to determine the molecular size of the antigen of the homogenates of *M. incognita* and *M. graminicola* recognized by western blot. Polymorphic bands were identified in Coomassie Blue stained gel with both the nematode species (Figure 4). 5 prominent bands of 87, 41, 32, 28 and 18 kDa specific to *M. incognita* and 3 distinct bands of 87, 41 and 33 kDa specific to *M. graminicola* was picked out for proteomics study. Several of the individual peptides obtained from the MALDI-MS

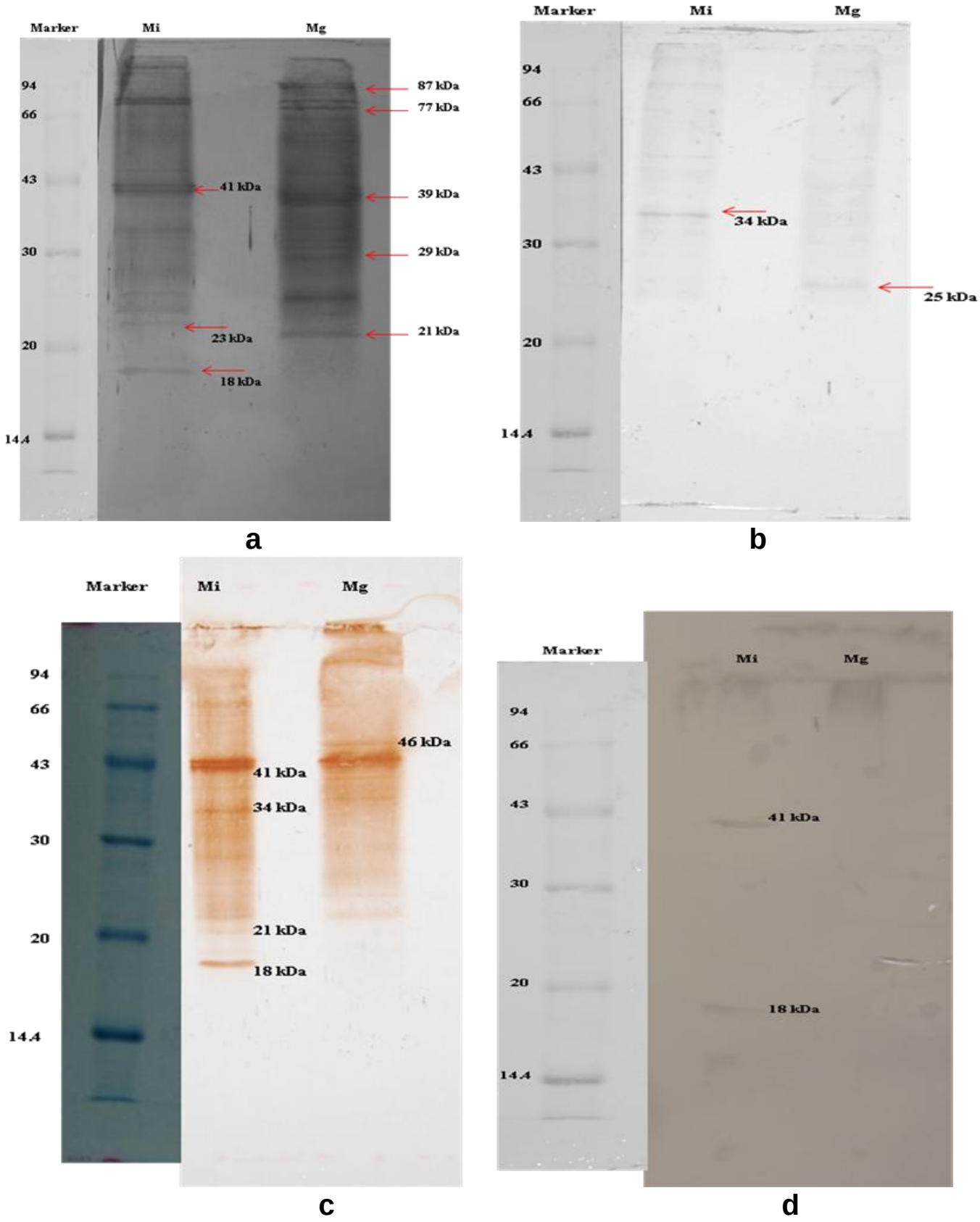

Figure 3. Western blot of *M. incognita* (Mi) and *M. graminicola* (Mg) stylet secreted proteins probed with a) PAb IACR-PC 374, b) PAb IACR-PC 389, c) PAb IACR-PC 373, d) MAb IACR-CCNj.2a.15.

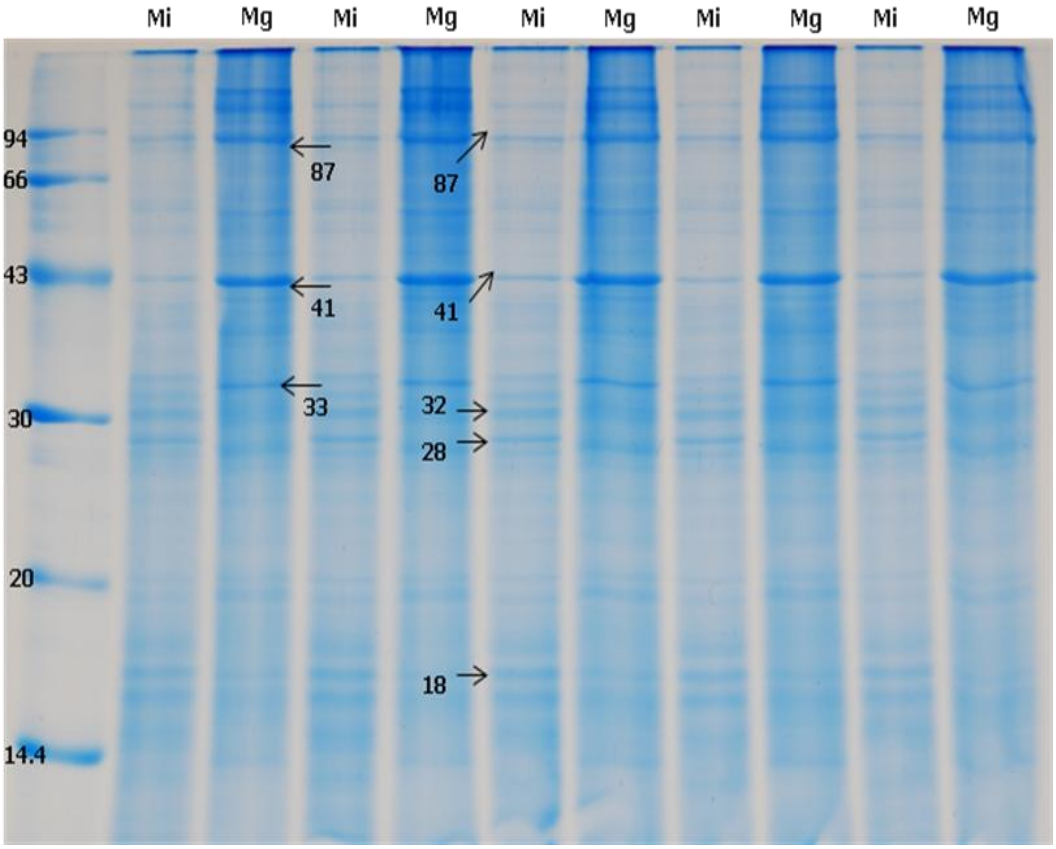

Figure 4. Analysis of homogenate proteins of *M. incognita* (Mi) and *M. graminicola* (Mg) by SDS-PAGE. Protein bands were visualized by Coomassie blue G250.

and ESI-MS data showed very high level of sequence similarity to the signal recognition particle protein, transmembrane protein, zinc finger motif, galactose binding lectin, neurotransmitter gated ion channel proteins, cellulose binding precursor and FMRFamide-like peptides of several nematode species using protein Blast search in Genbank (Table 1).Galactose binding lectin or Galectin of *M. incognita* (Minc03540) which is 308 amino acids long were chosen for further study as it may play the imperative role during the host recognition process. Theoretical isoelectric point (pI) and molecular weight (mw) for the conceptually translated protein (Minc03540) was calculated by the Expasy Protparam tool showing mw of 35490 dalton and pI of 5.33. The instability index (36.73) classified it as stable protein. SignalP result predicted 21 residue long signal peptide at N-terminal end, suggesting the protein has excellular function like signal transduction. Galectin protein sequences of closely related species were retrieved from Genbank database using protein Blast Search. Best hits were obtained with *Caenorhabditis elegans*, *Caenorhabditis briggsae* and *Brugia malayi*. The homologous sequences aligned using multiple sequence alignment (MSA) suggested that galectins are highly conserved across the nematode genera regardless of amino termini or carboxyl termini (Figure 5).

The predicted secondary structure of Minc03540 revealed that it has more number of beta sheets as compared to alpha-helices revealing the protein is folded properly (Figure 6). The predicted C-score (-0.69) indicated the model is of higher confidence and TM-score (0.63) signifying the model is in correct topology. To analyze the stereochemical quality of the predicted structure PROCHECK software was used. According to PROCHECK results, the first model (Table 2) seems to be most appropriate one because it has most of the amino acid residues present in the core and allowed regions (95.9%) while only 3.0% of the total amino acids were found in the generous region as indicated by Ramachandran plot (Figure 7). Further, the quality was assessed by the VERIFY 3D server which verified the 3D structural distribution of amino acids as compared to the 1D distribution of amino acid residues. According to the results provided by the DoBo server (http://sysbio.rnet.missouri.edu/dobo) the N-terminal domain stretches from amino acid 1-46 and the C-terminal domain stretches from amino acid 192-308.

The model has shown structural similarity with several

Table 1. Individual peptides obtained from MALDI-MS and ESI-MS data showed sequence homology to the target proteins of several nematode species from the genomic database.

Protein spot	Micro-sequence	Homology with known proteins (% of identical amino acids)
M. incognita		
Spot 1.	MSHPGWIMVSFLTELLSQSSK	100% neurotransmitter gated ion channel protein of *C. elegans*
87 kDa	WSFYLTSLSEYFDEDVNIDQP	90% neurotransmitter gated ion channel protein of *C. elegans*
Spot 2.	MANKLIPVLLIFFLSVPQFDAD	90% galectin (Minc03540) protein of *M. incognita*
41 kDa	LEVDGTFELHTVTINNAR	80% galectin (Minc03540) protein of *M. incognita*
Spot 3.	MFFVLILLFSFPPFCFPNKFSSK	100% zinc finger motif (Minc02576) of *M. incognita*
32 kDa	RFQHERDLYYFTMSHLGNLG	85% zinc finger motif (Minc02576) of *M. incognita*
Spot 4.	MSGCLDQIRCNCTFDLEGRRN	75% transmembrane protein of *C. briggsae*
28 kDa	SSTILGVYFPFVALFLFRFI	70% transmembrane protein of *C. briggsae*
Spot 5.	MVLADLGRKIRNAISKL	80% signal recognition particle protein of *Brugia malayi*
18 kDa	LQNMMKQLQGASSSLGNRRN	95% signal recognition particle protein of *Brugia malayi*
M. graminicola		
Spot 6.	ASFFYLLIISVSLLILANADDA	88% cellulose binding precursor of *M. javanica*
87 kDa	VENRDIGVVYNDVPEPLPTI	60% cellulose binding precursor of *M. javanica*
Spot 7.	LALFGFVVLIVGQMSVLGA	95% FMRFamide-like peptides of *M. incognita*
41 kDa	SSGGNKGNNFLRFGR	65% FMRFamide-like peptides of *M. incognita*
Spot 8.	MSIFLTSALLIISLIAMTEG	60% msp1 gene of *M. incognita*
33 kDa	VDFKIVPTDKKISPKACTMKM	85% msp1 gene of *M. incognita*

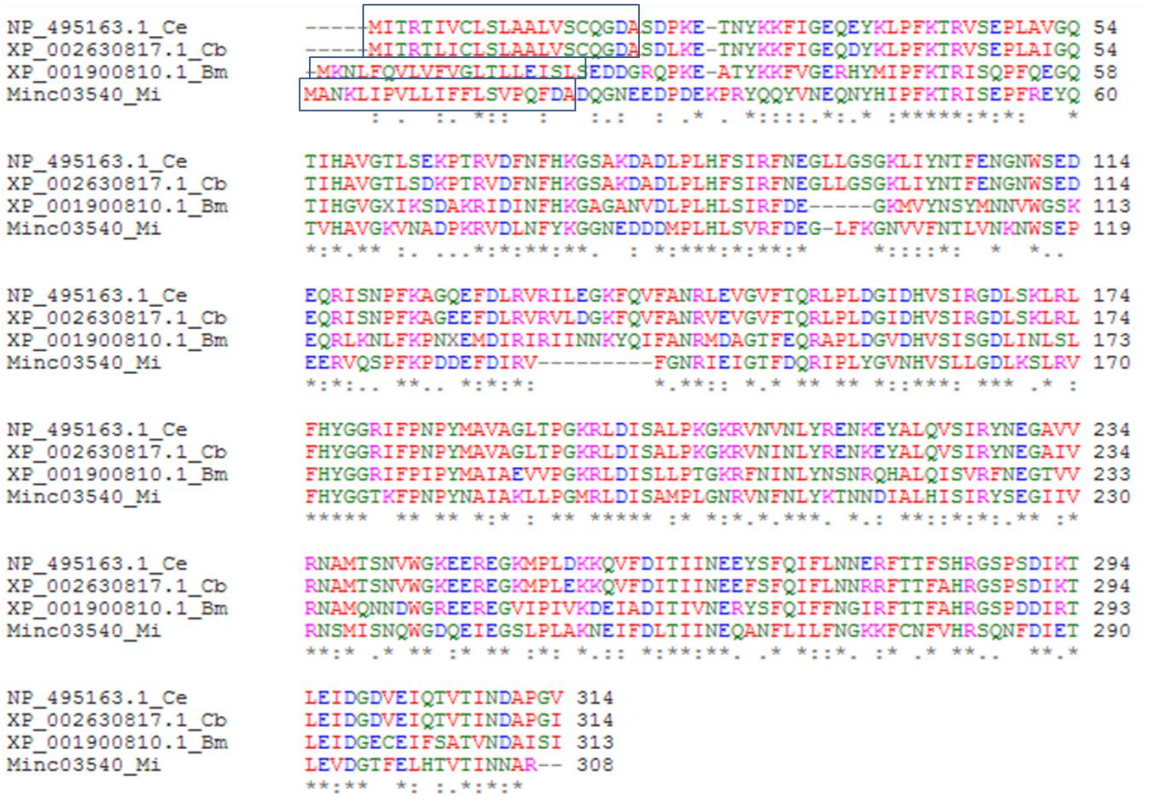

Figure 5. MSA of the predicted amino acid sequence of galectin of *M. incognita* with *galectin* sequences from other nematodes. The sequences are denoted by their Genbank identifier followed by the species abbreviation. Ce, *Caenorhabditis elegans*; Cb, *Caenorhabditis briggsae*; Bm, *Brugia malayi*; Mi, *Meloidogyne incognita*. * and **:** signs indicates conserved and similar amino acids respectively. Boxed region represents signal peptide.

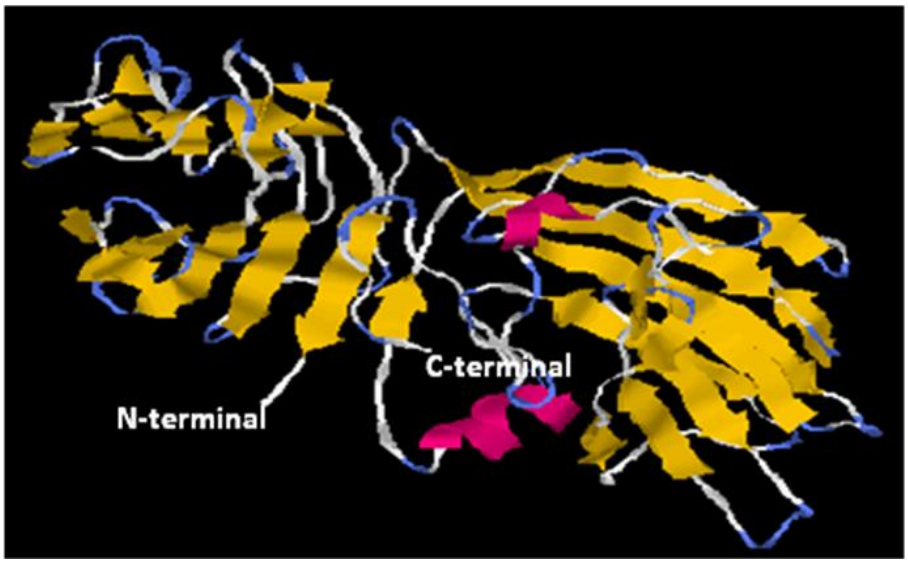

Figure 6. Predicted secondary structure of Minc03540 protein of *M. incognita* using *ab initio* protein modelling server I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER).

Table 2. Evaluation results of the I-TASSER models of the tertiary structure by PROCHECK and VERIFY 3D. *****These models had an average 3D-1D score >0.2.

Parameter		Model 1 (%)	Model 2 (%)	Model 3 (%)	Model 4 (%)	Model 5 (%)
PROCHECK	Core Region	80.0	72.2	77.0	70.0	69.6
	Allowed Region	15.9	18.9	16.3	20.7	22.6
	Generous Region	3.0	6.3	5.2	4.8	4.8
	Disallowed Region	1.1	2.6	1.5	4.4	3.0
*VERIFY3D		67.0	64.0	71.0	65.0	70.0

concanavalin A-like lectins/glucanases from the PDB database. Further, gene ontology terms suggested that the protein has carbohydrate binding affinity (GO: 0005529). The protein has more number of positively charged residues (aspartate and glutamate) as compared to negatively charged ones which is suspected to interact with the cations emanating from rhizosphere during host recognition process.

Conclusion

The antigens identified in this study might represent potential targets for nematode control, since proteins from SC and SE products are the first molecules to have contact with their hosts, and, therefore, might play an important role in the host-parasite interaction including host recognition, invasion and development processes. Problems have been encountered in obtaining resistance sources against a particular pest/pathogen and emergence of resistance breaking pathogen races or biotypes. These problems can now be overcome by using modern day cellular and molecular approaches to plant biotechnology, e.g. RNAi based transgenics which can facilitate the transfer of existing sources of nematode resistance across conventional barrier to reproduction into other related or even unrelated crop species.

Furthermore, molecular analysis of nematode/host interaction and molecular dissection of nematode systems such as neurobiology, sensory perception or moulting, may now allow the construction and expression in plant of novel broad spectrum form with synthetic resistance. This investigation was an attempt to generate more information at molecular level with respect to interactions of nematodes and host plants in the areas of nematode host finding.

Work is in progress to characterise the identified proteins other than galectin and their role in plant-nematode interaction to be established by knocking out those genes by RNA interference.

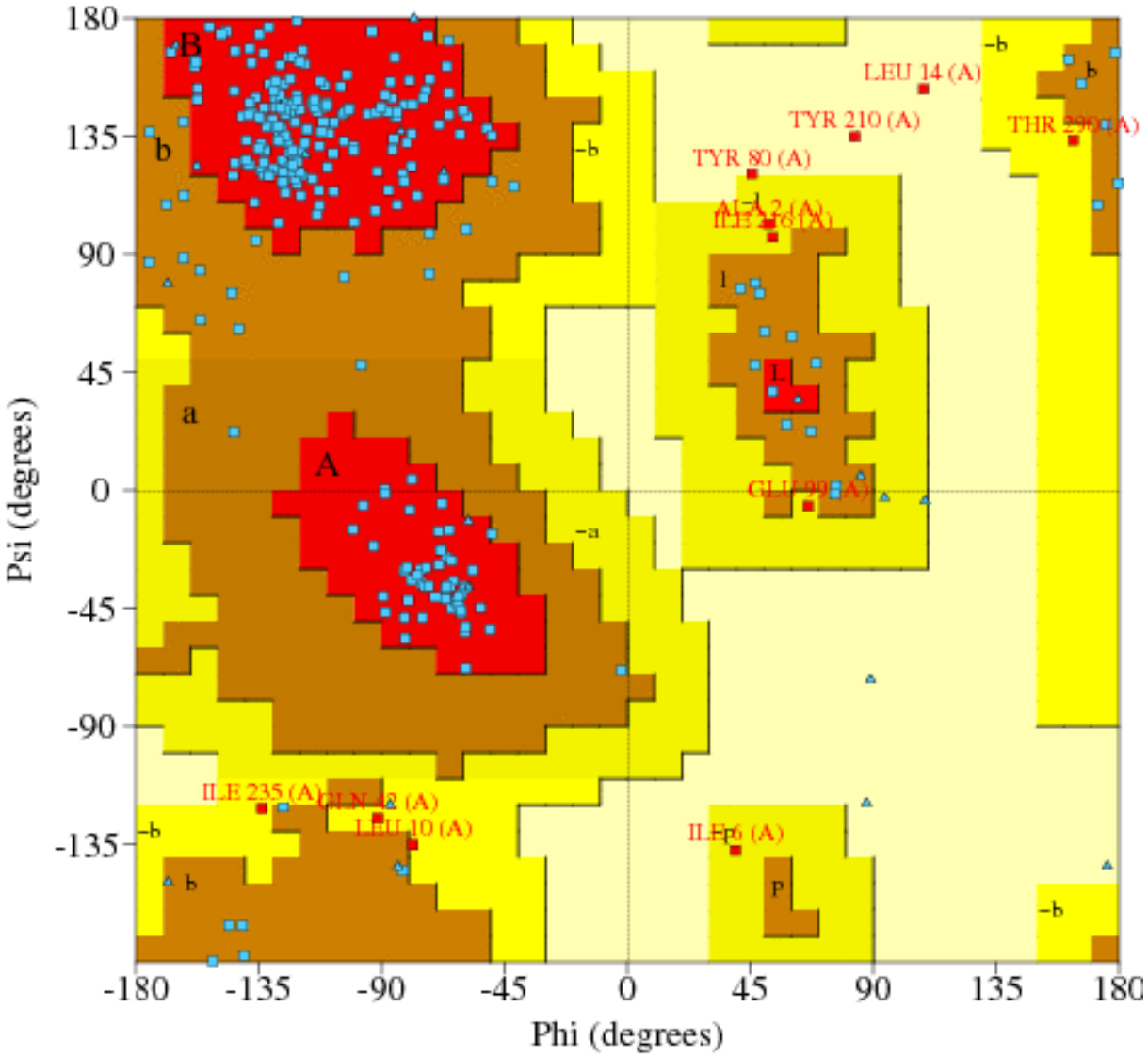

Figure 7. Ramachandran plot of the predicted model of Minc03540 protein of *M. incognita* showing Psi and Phi angles.

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