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Full Length Research Paper

Distribution and pathotype identification of *Xanthomonas citri* subsp. *citri* recovered from south-western region of Saudi Arabia

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We investigated the distribution of *Xanthomonas citri* subsp. *citri* (*Xcc*) pathotypes in south-western region of Saudi Arabia. A total of 76 *Xanthomonas* like strains were isolated from different citrus species showing bacterial canker symptoms from citrus commercial farms, backyard orchards and nurseries. These strains were subjected to biochemical, molecular and pathogenicity tests on leaves of grapefruit (*Citrus paradisi*). Based on symptoms induced on leaves of grapefruit, these strains were divided into two groups. There were 21 strains that induced typical erumpent canker lesions with water-soaked margin, belonging to *Xcc* type A. On the other hand, strains that produced flat necrotic lesions with water-soaked margin on grapefruit leaves belonged to *Xcc* A* type (55 strains). The physiological and biochemical tests, ImmuneStrip ® assays and 16S rDNA analysis confirmed the identity of the two *Xcc* pathotypes: A and A*. Our results indicate that, the two pathogenic variants co-exist in south-western region of Saudi Arabia and this could further favour the generation of new genetic variants through recombination and horizontal genetic exchange. The generation of new aggressive pathotypes may threaten the citrus plantation in Saud Arabia.

Key words: Disease diagnosis, pathogenicity, citrus bacterial canker.

INTRODUCTION

Citrus bacterial canker (CBC) caused by *Xanthomonas citri* subsp. *citri* (*Xcc)* (Schaad et al., 2006), is an important disease in many citrus production areas world-wide including Saudi Arabia (Gottwald et al., 2002; Ibrahim and Bayaa, 1989). The pathotypes of the casual agent are primarily separated by their geographical origin, host range and certain genotypic characteristics (Brunings and Gabriel, 2003). The common well-known pathotypes are A, B, and C. The citrus canker B (cancrosis B or false canker) was reported in Argentina, Uruguay and Paraguay on lemon (*Citrus limon*) (Schubert et al., 2001). The C type of citrus canker (cancrosis C) is also caused by *X. citri* pv. *aurantifolii*. It has been found only in São Paulo state in Brazil and it has only two known hosts; Mexican lime and sour orange. The symptoms induced by the three forms of canker are similar and induce erumpent and corky lesions surrounded by oily or water-soaked margins on leaf, fruit, and stem tissues (Civerolo, 1984). The Asiatic form or pathotype A of *Xcc* is widespread throughout the world and affects the widest range of citrus hosts (Schubert et al., 2001; Juhasz et al., 2013; Hoarau et al., 2013). However, several groups within pathotype A with restricted host range have been identified. Group A* was reported for the first time by Vernière et al. (1998) in Southwest Asia. This group was isolated from Mexican lime trees in several countries in Southwest Asia; including Saudi Arabia, Oman and Iran. The pathogen produces typical

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erumpent bacterial canker lesion on Mexican lime but not on grapefruit (Vernière et al., 1998). Recent studies reported the presence of group A* in Thailand, Cambodia, Ethiopia and Burkina Faso (Bui et al., 2007; 2008; Derso et al., 2009). In 2012, typical CBC symptoms were observed on leaves, stems and fruits of Mexican lime trees in south western region of Saudi Arabia where CBC had not been previously detected (personal observation). Interestingly, other CBC susceptible citrus trees near the infected ones (within 3 m of a diseased tree) did not show any CBC symptoms. Initial laboratory pathogenicity tests on grapefruit leaves revealed that there were two different types of symptoms produced by *Xcc* strains. The purpose of this study was to determine which strains of *Xcc* are present in south-western region of the Saudi Arabia, specifically the citrus major producing areas e.g. Al-Bahah, Aseer and Jazan.

MATERIALS AND METHODS

Surveyed areas

Ten locations belonging to three citrus-growing regional areas as mentioned before were surveyed for citrus bacterial canker strains distribution on commercial farms, backyard orchards and nurseries. The disease incidence of citrus canker for each block was calculated by expressing the number of diseased trees as a percentage of the total number of trees inspected. The severity of canker in each 5-tree block was rated visually on the following scale: 0 = no symptoms, 1 = isolated leaf lesions, 2 = lesions restricted to one side of the canopy, 3 = lesions distributed over the entire canopy and 4 = greater occurrence of leaf lesions than in 3 (Agostini et al., 1985).

Isolation of bacterial strains

Seventy six citrus samples with bacterial canker-like symptoms were collected from seventeen orchards located in various regions. Samples of diseased leaves, stems and fruits were collected from each orchard planted with either one variety or mixed varieties from four to five trees per row. Both sampled trees and rows were selected at random. Samples were sealed in plastic bags and forwarded to the quarantine laboratory at the King Saud University, College of Food and Agriculture Sciences, Plant Protection Department. Samples were washed under running tap water for 10 min. Infected areas were soaked in 1% sodium hypochloride solution for 30 s and rinsed in sterile distilled water three times. One lesion, and 2 mm of the peel around it, was cut into small pieces with a sterilized scalpel, comminute in Phosphate Buffer Saline (PBS, pH 7.2) and left for 10-20 min at room temperature. 100 µl of the PBS extract were streaked onto plates of nutrient agar media supplemented with 1% glucose (NGA) (Lelliot and Stead, 1987) and incubated at 28°C for 48-72 h. Bacterial colonies with yellow pigmentation were picked up and transferred to new NGA plates for purification and further tests.

Pathogenicity tests

Inoculum preparation

Isolates of *Xcc* were grown on NGA plates and incubated at 28°C for 24 h. Bacterial cells were suspended in sterile distilled water and the bacterial suspension was adjusted to 10^8 CFU ml^{-1} (OD_{660}= 0.06).

Detached leaves

Pathogenicity of purified *Xcc* strains was evaluated on detached grapefruit leaves cv. Duncan. Surface of young leaves was disinfected with 70% ethanol, washed with sterile water and placed on the surface of 1% water agar with their abaxial surfaces facing upwards (Vernière et al., 1991). Ten wounds per leaf were performed with a needle and droplets (10 µl) of bacterial suspensions of 1×10^8 CFU $ml^{-}1$ were placed on each wound. Leaves were incubated for 1-2 weeks at 28°C until symptoms appearance. Negative controls had leaves treated with sterile water.

Attached leaves

Grapefruit cv. Duncan fully expanded leaves were infiltrated by pressing the opening of a syringe without a needle gently against the abaxial leaf surface supported by one finger with 1×10^8 CFU $ml^{-}1$ of *Xcc* strains (Vernière et al., 1991). Plants were maintained in the greenhouse at 28-30°C.

Physiological and biochemical characterization

A total of 76 strains were identified according to tests described by Fahy and Persley (1983), Lelliott and Stead (1987) and Vernière et al. (1991).

ImmuneStrip ® assay

ImmuneStrip assay was conducted according to the instruction in the manual of *Xcc* immuneStrip ® test proposed by Agdia, Inc. (Catalog No. STX92200). Bacterial suspensions were diluted with the immunoStrip buffer. The strips were dipped into the immunoStrip buffer for 5 min until both the control line and the test line appeared. Positive results gave both control and test lines, while negative results gave only the former one.

Amplification and sequencing of 16S rDNA gene

DNA extraction

Total DNAs of *Xanthomonas* strains were extracted using the method described by Llop et al. (1999). The DNA was stored at -20°C until further use for PCR.

Sequence analysis

Amplification of the 16S rDNA was done by using primers 27F and 1492R (Lane, 1991). The PCR products were sent to the Advanced Genetic Technologies Centre, University of Kentucky, Lexington, USA to be cleaned and sequenced. DNA sequences were cleaned and alignment using BioEdit Software and the cleaned sequences were searched against other 16S rDNA sequences deposited in the NCBI GenBank database.

Statistical analysis

In cases where disease incidence data were not normally distributed, analysis of variance and separation on means was performed

Table 1. Strain designation, host plant, location and pathotype identification of *Xanthomonas citri* subsp. *citri* strains recovered from south-western of Saudi Arabia.

Number of isolate	Strain designation	Host plant	Region	Pathotype identification	Accession number
12	Aseer 1-12	*C. sinensis*	Aseer	A	JQ890091*
15	Aseer 13-27	*C. aurantifolia*	Aseer	A&A*	JQ890092
20	Bah 1-20	*C. aurantifolia*	Al-Bahah	A&A*	JQ890093
5	Jaz 1-5	*C. limon*	Jazan	A	JQ890094
24	Jaz 6-29	*C. aurantifolia*	Jazan	A*	JQ890095

*No nucleotide polymorphisms was detected among the 16S rDNA region of Saudi *Xcc* strains.

Table 2. Distribution, incidence and severity percentage of canker disease caused by *Xanthomonas citri* subsp *citri* on citrus leaves in south-western region of Saudi Arabia.

Region	locations	Variety	incidence (%)[1]	Severity (%)[2]	Citrus trees age (year)
Al-Bahah	Al-Bahah	Mexican lime	77abc	1.99b	10
	Al-Makhwah	Mexican lime	74c	1.83b	8
	Baljurshi	Mexican lime	74c	1.77b	8
Asser	Abha	Mexican lime	83ab	2.90a	6
	Mahail	Sweet orange	78abc	2.01ab	12
	Balqarn	Mexican lime	83ab	2.65ab	10
Jazan	Abu Arish	Mexican lime	84a	2.98a	10
	Jizan	Lemon	76abc	1.85b	8
	Sabya	Mexican lime	83ab	2.91a	8
	Samitah	Mexican lime	80b	2.35ab	10

[1] Average of 15 trees with canker symptoms; [2]Mean of five canker ratings (1 rating per 5 tree block) where 0 = no disease and 4 = sever (see text). Means within a colum followed by the same letters are not statistically different at $P \leq 0.05$ (Tukey test).

on arcsine transformed data, but percent data were reported. Disease severity data were analyzed according to the Kruskale Wallis nonparametric test statistics. Analysis of variance was performed and means were separated according to the Tukey test. The experiments were repeated twice.

RESULTS

Orchard survey and bacterial isolation

A total of seventy six *Xcc* strains was isolated from infected leaf, stem and fruit samples collected from symptommatic Mexican lime (59 strains), sweet orange (12 strains) and lemon (5 strains) plants from different locations (Table 1). Bacterial citrus canker was detected in all citrus growing regions surveyed in Saudi Arabia (Table 2). Typical canker symptoms were observed not only on leaves but also on fruits and twigs of citrus trees (Figure 1). Also, water soaked regions around the raised corky lesions on the fruits were observed. No chlorosis was observed around the corky lesions on twigs. The overall disease incidence on leaves was 79.2% with at least one lesion per leaf. Disease severity expressed as percent infected leaf area was 32.2%. The highest disease incidence was recorded in Jazan on Mexican lime followed by Asser, Balqarn and Sabya, respectively. Canker severity on Mexican lime recorded in Abu Arish (2.98%) was significantly higher than in Baljurshi (Table 2). Based on the field host range and pathogenicity tests, it appeared that the *Xcc* variant that occurred in all locations of Al-Bahah, one location in Aseer and two locations in Jazan regions had similar host ranges to that of the atypical Asiatic (*Xcc*-A*) form.

Pathogenicity tests

Based on pathogenicity tests on grapefruit leaves, all strains were divided into two groups. In detached leaves, bacterial strains gave two different kinds of symptoms. Strains of group one gave erumpent callus-like lesions which surrounded by thin water soaked margins (Figure 2a), while the second group caused no symptoms post inoculation (Figure 2b). In case of attached leaves, the first group strains was pathogenic and showed water soaking regions following hyperplasia and hypertrophy and necrosis (*Xcc*-A) (Figure 3a), while the second group (*Xcc*-A) gave only flat necrotic appearance on the infiltrated areas (Figure 3b). Hypertrophy and hyperplasia were observed 6 days after inoculation. The flat necrotic lesions were clear

Figure 1. Canker symptoms caused by *Xanthomonas citri* subsp. *citri Xcc*-A* strains on Mexican lime (a) and *Xcc*-A strains on Sweet orange (b) which bacteria were consistently isolated.

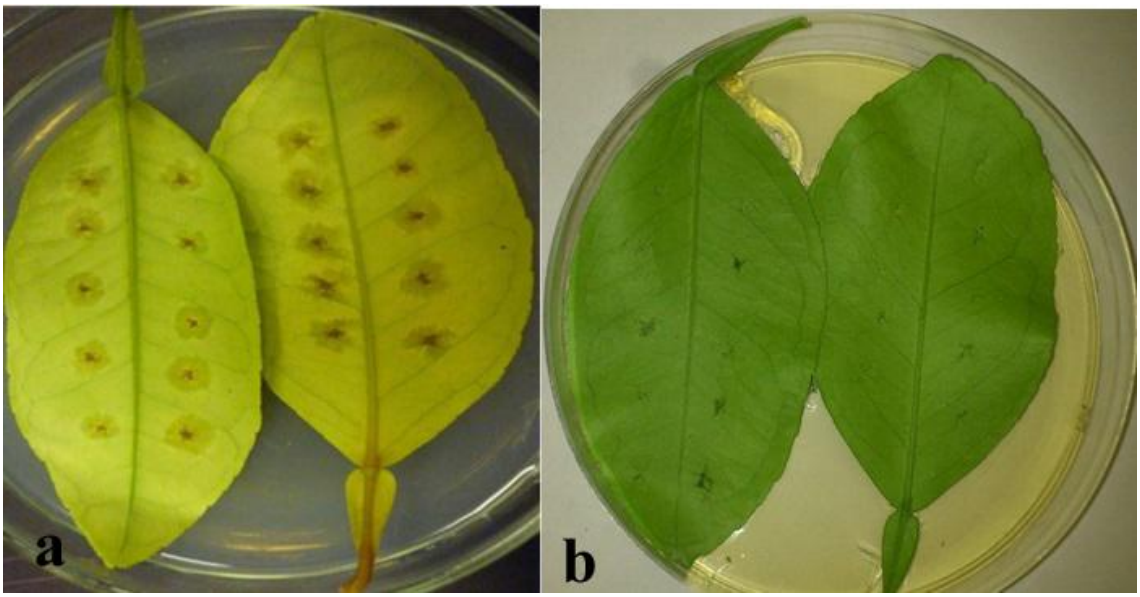

Figure 2. Response on Duncan grapefruit in a detached leaf assay after one week post inoculation with (a): *Xcc*-A strains, (b): *Xcc*-A* strains. All *Xcc*-A strains produced erumpent callus-like tissue with water-soaked. In contrast, the *Xcc*-A* strains caused no symptoms after inoculation.

and the hyperplasia developed well and resulted in necrotic regions. No symptoms were observed on control plants inoculated with sterile water in both inoculation methods. All *Xcc* strains were re-isolated from inoculated leaves and re-identified by phenotypic characters.

Physiological and biochemical characterization

Data in Table 3 show that all *Xcc* strains were Gram-negative; rod shaped, motile, aerobic, non-fluorescent on King's medium B, but grew with characteristic mucoidal colonies on media containing glucose. They were able to hydrolyze casein, gelatine, starch, Tween 20 and 80. In addition, all isolates had the ability to grow on media supplemented with 1, 2, and 3% NaCl. All the strains were oxidase negative, arginine-dihydrolase positive and did not macerated potato discs. Moreover, the strains utilised sucrose, cellubiose, L-rhamnose and L-arabinose. All strains grew at 36 and 4^{o}C but not at 40^{o}C. The identity of bacterial strains was also confirmed with the immunoStrips designed for detection of *Xcc*. Collectively, the biochemi-

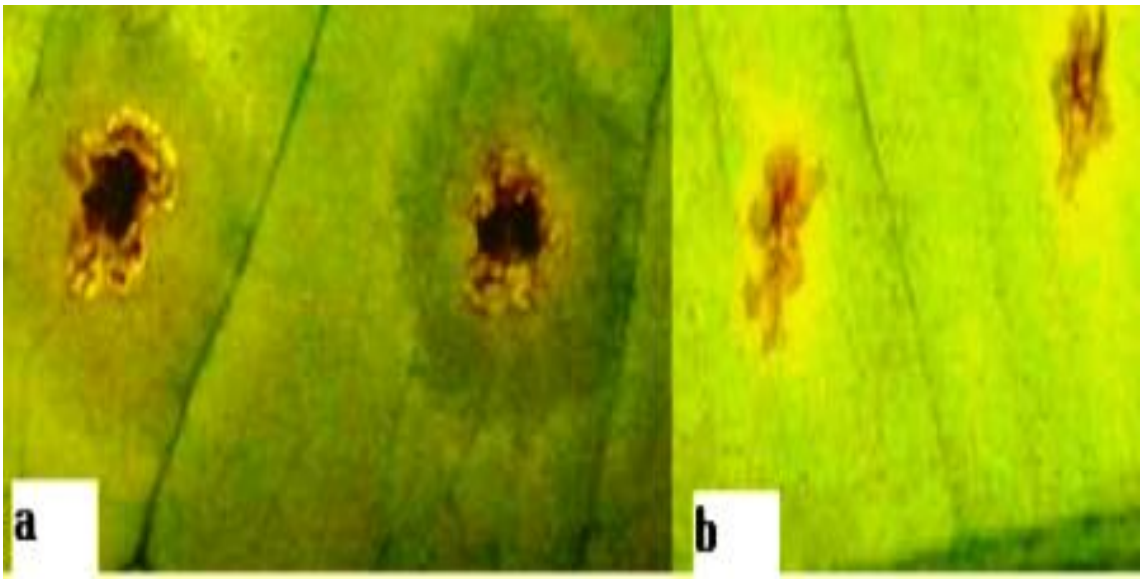

Figure 3. Symptomatology of *Xanthomonas citri* subsp. *citri* strains (A and A*) on attached grapefruit leaves cv. Duncan. Typical callus-like lesions with water soaked margins developed by *Xcc*-A while, b) flat less water soaked margin belonged to *Xcc*-A*.

Table 3. Biochemical and physiological characteristics of Saudi Arabia strains of *Xanthomonas citri* subsp *citri,* the causal agent of citrus canker disease.

Characteristic	Reaction
Gram reaction	-
Oxidative/fermentative tests	O
Arginine dihydrolase	-
Fluorescent pigmentation	-
Growth at 36°C	+
Growth at 40°C	–
H_2S generation from cysteine	+
Indole test	–
Levan production	+
Litmus milk test	Alkaline
MR-VP	–
NaCl tolerance: 1, 2 & 3% (w/v)	+
Nitrate reductase	–
Potato rot	+
Hydrolysis of: casein, gelatin, starch,Tween 20 and 80	+
Utilization of	
L-arabinose	+
L-rhamnose	+
Cellubiose	+
Sucrose	+

+ Positive reaction; -negative reaction.

cal identification of the strains confirmed their identity as *Xcc*.

Identification with 16S rDNA gene sequencing

The bacterial strains were also confirmed at the molecular level by sequencing 16S rDNA gene. The 16S rDNA analysis could not discriminate *Xcc* Saudi Arabian strains. Comparison between the partial sequences of 16S rDNA of Saudi strains and the other sequences of 16S rDNA deposited in GenBank showed that isolates were *Xcc.*

DISCUSSION

In our study, we aimed to determine the distribution of *Xcc* strains in the main citrus-growing areas of Saudi Arabia. Our survey showed the presence of *Xcc*-A and/or A* strains in the visited orchards with different extent of infection. During the survey, citrus canker was found on Mexican lime, sweet orange and lemon varieties. In all Al-Bahah locations, two locations in Jazan and one location in Aseer, other citrus varieties did not show any citrus canker symptom and that, hence, indicated the host specificity of the causal agent. The fact that, citrus canker disease was observed in commercial farms, backyard orchards and nurseries might indicate that the pathogen was probably introduced with the plant material used in citrus plantations. In addition, all the surveyed locations for canker disease used the flood irrigation system. The use of this system and contaminated seedlings might have disseminated citrus canker in these areas. The group A* was reported for first time with the same symptoms on Mexican lime by Vernière et al. (1998) in Southwest Asia and in Thailand and Cambodia (Bui et al., 2007; 2008) and Ethiopia (Derso et al., 2009), while, the group A^w strains produced brownish, flat and necrotic lesion on grapefruit (Sun et al., 2004).

Vernière et al. (1998) used several biochemical tests to identify and differentiate different pathotypes of citrus canker bacteria. The tests included hydrolysis of gelatine and casein, in addition to the growth on 3% NaCl. They reported that citrus canker pathotype A gave positive results on the three previous tests, while pathotype B had negative results for these tests. Patothype C gave a positive result only on hydrolysis of casein. In the present study, all bacterial isolates recovered from Saudi Arabia had the ability to hydrolyze both casein and gelatin and grew on media supplemented with 3% NaCl. We concluded that these tests were not able to discriminate between the Saudi Arabia strains. Vernière et al (1998) also reported that phenotypic tests based on carbon source utilization usually do not discriminate *Xcc*-A* strains from *Xcc*-A. In order to differentiate between groups of pathotype such as A, A^w and A* another test is needed. Generally, bacterial strains in this study were identified as *Xcc* according to Fahy and Persley (1983); Schaad (1988) and immunoStrip test. To confirm the identity of different Saudi strains causing citrus canker disease, the 16S rRNA sequences showed that both strains were *Xcc* with a similarity of 99%. Moreover, the analysis of 16S rDNA sequences did not give any discrimination between Saudi strains. Our results corroborate with the previous research that showed 16S rDNA sequences of different pathotypes of *Xanthomonas* causing citrus canker disease (A, B and C) were *Xcc* with a similarity of 99% (Lee et al., 2008).

Fox et al. (1992) reported that the 16S rDNA gene was considered unsuitable for discriminating and identifying closely related strains due to the high levels of sequence similarity in this region.

The results of our study based on physiological, biochemical, genetic analyses and pathogenicity tests showed that two strains (*Xcc*-A and *Xcc*-A*) were associated with bacterial citrus canker in Saudi Arabia. Identification of both pathotypes in citrus nurseries in Saudi Arabia suggests that there is a potential large scale distribution of these strains within the citrus orchards in this region. As diseased citrus nursery plants are a major source of primary inoculum, sanitation of citrus nurseries against citrus canker in Saudi Arabia is a prerequisite for improving disease management.

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REFERENCES

Agostini JP, Graham JH, Timmer LW (1985). Relationship between development of citrus canker and rootstock cultivar for young 'Valencia' orange trees in Misiones, Argentina. Proc. Fla. State. Hortic. Soc. 98:19-22.

Bruning AH, Gabriel DW (2003). *Xanthomonas citri*: breaking the surface. Mol. Plant. Pathol. 4:141–157.

Bui Thi Ngoc l, Verniere C, Pruvost O (2007). First report in Thailand of *Xanthomonas axonopodis* pv. *citri*-A* causing citrus canker on Lime. Plant Dis. 91:771.

Bui Thi Ngoc l, Verniere C, Pruvost O (2008). First report of *Xanthomonas citri* pv. *citri* causing citrus canker in Mali. Plant Dis. 92:977.

Civerolo EL (1984). Bacterial canker disease of citrus. J. Rio. Grande. Valley. Hortic. Soc. 37: 127-146.

Derso E, Verniere CJ, Pruvost O (2009). First report of *Xanthomonas citri* pv. *citri* A* causing citrus canker on Lime in Ethiopia. Plant Dis. 93: 203.

Fahy PC, Persely GJ (1983). Plant Bacterial Diseases. A diagnostic guide. London, Academic Press, New York.393pp.

Fox GE, Wisotzkey JD, Jurtshuk Jr. P (1992). How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. Int. J. Syst Evol. Microbiol. 42: 166–170.

Gottwald TR, Graham JH, Schubert TS (2002). Citrus canker: The pathogen and its impact. Plant Health Progress. [http://www.plantmanagementnet work.org/pub/php/review/citruscanker /].

Hoarau J, Boyer C, Vital K, Chesneau T, Verniere C, Roux-Cuvelier M, Pruvost O (2013). First report of *Xanthomonas citri* pv. *citri*-A causing Asiatic citrus canker in Mayotte. Plant Dis. 97:989.

Ibrahim G, Bayaa B (1989). Fungal, bacterial and nematological problems of citrus, grape and stone fruits in Arab countries. Arab. J. Pl. Prot. 7: 190–19.

Juhasz CC, Leduc A, Boyer C, Guerin F, Verniere C, Pruvost O (2013). First report of *Xanthomonas citri* pv. *citri* causing Asiatic citrus canker in Burkina Faso. Plant Dis. 97:1653.

Lane DJ (1991). 16S/23S rDNA sequencing, In: Stackebrandt E. and Goodfellow M (eds), Nucleic acid techniques in bacterial systematic, Chichester, England, John Wiley and Sons:115-175.

Lee YH, Lee S, Lee DH, Ji SH, Chang HY, Heu S, Hyun JW, Ra D-S, Park EW (2008). Differentiation of citrus bacterial canker strains in Korea by host range, rep-PCR fingerprinting and 16S rDNA analysis. Eur. J. Plant Pathol. 121:97-102

Lelliott RA, Stead DE (1987). Methods for diagnosis of bacterial disease of plants. Methods in Plant Pathology Vol. 2, Ed. T.F. Peerce. Blackwell Scientific Publication, 216pp.

Llop P, Caruso P, Cubero J, Morente C, Lopez MM (1999). A simple extraction procedure for efficient routine detection of pathogenic bacteria in plant material by PCR. J. Microbiol. Meth. 37: 23-31.

Lou BH, Deng CL, Song YQ, Zhao XL, Wang MZ, Li XL (2013). First report of *Xanthomonas citri* subsp. *citri* pathotype a causing citrus canker on *Nanfeng Tangerine* in Guangxi Province, China. J. Plant Pathol. 95:664.

Schaad NW, Postnikova E, Lacy G, Sechler A, Agarkova I, Stormberg PE, Stormberg VK, Vidaver AK (2006). Emended classification of Xanthomonad pathogens on citrus. Syst. Appl. Microbiol. 29: 690–695.

Schaad NW (1988). Laboratory Guide for Identification of Plant Pathogenic Bacteria. American Phytopathological Society Press, Saint Paul, MN, 164 pp.

Schubert T S, Rizvi SA, Sun X, Gottwald T R, Graham JH, Dixon WN (2001). Meeting the challenge of eradicating citrus canker in Florida again. Plant Dis. 85:340-356.

Sun X, Stall RE, Jones JB, Cubero J, Gottwald TR, Graham JH, Dixon WN, Schubert TS, Chaloux PH, Stromberg VK, Lacy GH, Sutton BD (2004). Detection and characterization of a new strain of citrus canker bacteria from Key/Mexican Lime and alemow in South Florida. Plant Dis. 88: 1179-1188.

Vernière C, Devaux M, Pruvost O, Couteau A, Luisett J (1991). Studies on the biochemical and physiological variation among strains of *Xanthomonas campestris* pv. *citri* the causal agent of citrus bacterial canker disease. Fruits. 46: 160-170.

Vernière C, Hartung JS, Pruvost OP, Civerolo EL, Alvarez AM, Maestri P, Luisetti J (1998). Characterization of phenotypically distinct strains of *Xanthomonas axonopodis* pv. *citri* from Southwest Asia. Eur. J. Plant. Pathol. 104: 477-487.

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Full Length Research Paper

Pichia spp. yeasts from Brazilian industrial wastewaters: Physiological characterization and potential for petroleum hydrocarbon utilization and biosurfactant production

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Pichia **strains isolated from industrial wastewaters were physiologically characterized and tested for their potential for hydrocarbon utilization and biosurfactant production using hydrophobic and hydrophilic substrates. The emulsification index and growth curves were obtained to establish the relation between cell growth and this index.** ***Pichia anomala*** **CE009 grew in the range of 18 to 39°C and the optimum was 24°C. The strain was able to grow in NaCl concentrations between 2 and 15%, and pH values between 2 and 12.** ***Pichia membranaefaciens*** **CE015 grew at temperatures of 18 to 42°C and the optimum was 30°C. This strain grew in 2 to 10% of NaCl and pH from 2 to 12. Hexadecane, kerosene and diesel oil were used for growth but not for biosurfactant production, while glucose and glycerol were used for growth and biosurfactant production. Biosurfactant was detected during the exponential growth phase, with production peaks of 63% for** ***P. anomala*** **CE009 and 58% for** ***P. membranaefaciens*** **CE015. This study shows the potential of two** ***Pichia*** **strains for bioremediation exploitation under a wide range of environmental conditions.**

Key words: Emulsifying agents, *Pichia anomala*, *Pichia membranaefaciens*, glycerol.

INTRODUCTION

Yeasts have emerged as an important group with significant biological relevance and environmentally relevant applications. The great advantage of using yeasts is the generally regarded as safe (GRAS) status that most yeast species present. Organisms with GRAS status are not toxic or pathogenic; a fact that increases the range of possible uses such as for biodegradation and biosurfactant production (Fontes et al., 2012).

Kerosene and diesel oil, composed by fractions of aromatic and aliphatic hydrocarbons, are examples of environmental pollutants (Saratale et al., 2007), while the hexadecane (HXD), present in the aliphatic fraction of crude oil (Chénier et al., 2003), has been used as a model molecule to study aliphatic hydrocarbon utilization (Schoefs et al., 2004).

Conventional chemical surfactants are organic substances composed of hydrophilic and hydrophobic portions. These compounds are commonly used to separate oily materials from a particular medium because they are able to increase the aqueous solubility of non-aqueous phase liquids (NAPLS) by reducing their interfacial tension at the air-water and water-oil interfaces (Yin et al., 2009). These compounds are costly and pose potential threats to the environment due to their recalcitrance and toxicity.

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Tightening environmental regulations and increasing awareness of the need to protect the ecosystem have resulted in growing interest in biosurfactants as possible alternatives to synthetic ones (Banat et al., 2010). When properly used, biosurfactants are comparable to traditional chemical analogues in terms of performance and offer advantages of low toxicity, high biodegradability, high foaming capability, higher selectivity, specific activity at extreme temperature, pH and salinity, ability to be synthesized from renewable substrates and ecological acceptability (Fontes et al., 2008). Their environmental uses are related principally to the bioremediation of petroleum hydrocarbons in groundwater and soil and the breakdown of hazardous compounds (Coimbra et al., 2009). The addition of biosurfactants increases the availability of long-chain hydrocarbons to microbes and renders them more accessible to microbial enzyme systems for utilization (Khopade et al., 2012a, b).

Although the best known biosurfactants are of bacterial origin, as reported by Abouseoud et al. (2008), Wu et al. (2008), Das et al. (2009), Pansiripat et al. (2010), Abbasi et al. (2012), Eddouaouda et al. (2012), Khopade et al. (2012a, b), Sousa et al. (2012) and Burgos-Díaz et al. (2013), the study of biosurfactant production by yeasts has been growing in importance, with production being reported mainly by the genera *Candida* sp., *Pseudozyma* sp. and *Yarrowia* sp. (Morita et al., 2007, 2008; Ilori et al., 2008; Luna et al., 2009; Batista et al., 2010; Daverey and Pakshirajan, 2010; Luna et al., 2011, 2012; Accorsini et al., 2012; Fontes et al., 2012; Rufino et al., 2012; Chung et al., 2013). *Pichia* spp. are very interesting yeasts from the standpoint of environmental applications (Walker, 2011), but works on biosurfactant production by this genus are still scarce. Indeed, one study with *Pichia anomala* PY1, was done by Thaniyavarn et al. (2008) and another with *Pichia jadinii* by Dziegielewska and Adamczak (2013). Thus, the investigation of the potential of *Pichia* species to produce biosurfactants is an innovative aspect of this study.

The carbon source is a limiting factor in the production costs of biosurfactants (Abouseoud et al., 2008; Das et al., 2009). A possible strategy to reduce these costs is the use of alternative substrates, such as agricultural and industrial wastes. Some examples of residue substrates are different types of used oils, waste from the processing of plants such as molasses and recently glycerol, a residue from the production of biodiesel (Silva et al., 2009; Batista et al., 2010; Dobson et al., 2012; Luna et al., 2012).

Microbial applications are subject to changing environmental conditions, such as salinity, temperature and pH, to which the microorganisms must adapt in order to survive. In this sense, the present work aimed to evaluate the effects of pH, temperature and salinity on growth of *P. anomala* CE009 and *Pichia membranaefaciens* CE015 and to test the potential of these strains for petroleum hydrocarbon utilization and biosurfactant production using hydrophilic and hydrophobic substrates as carbon and energy sources.

MATERIALS AND METHODS

Yeasts

P. anomala CE009 and *P. membranaefaciens* CE015 were obtained from the culture collection of the Environmental Microbiology Laboratory of the Biology Department of Federal University of Ceará, Brazil. These strains are maintained at 4°C on potato dextrose agar (PDA) (Oxoid) covered with mineral oil. *P. anomala* CE009 was isolated from a cashew nut processing plant effluent and *P. membranaefaciens* CE015 from an oil refinery effluent, both in Fortaleza, Ceará, Brazil. The strains were previously identified by conventional methods used in yeast taxonomy (Kurtzman and Fell, 1998).

Organic substrates

Kerosene and diesel oil were supplied by the oil company Petrobras. Glycerol, glucose and hexadecane were provided by Sigma-Aldrich (USA).

Inoculum standardization

The strains *P. anomala* CE009 and *P. membranaefaciens* CE015 were grown in yeast malt agar (YMA) medium at 25°C for 48 h. Then, three colonies from of each strain were transferred to 250-mL Erlenmeyer flasks containing 50 mL of yeast malt broth (YMB) composed of peptic digest of animal tissue 5.0 g L^{-1}, yeast extract 3.0 g L^{-1}, malt extract 3.0 g L^{-1}, dextrose 10.00 g L^{-1}, final pH (at 25 °C) of 6.2 ± 0.2. The flasks containing the strains were incubated in a rotary shaker at 150 rpm at 25°C for 24 h, after which the optical density (OD) of the cultures were adjusted to approximately 0.5 at 600 nm, corresponding to a density of 10^6-10^7CFU mL^{-1} according to the calibration curve of CFU mL^{-1} versus OD.

Physiological characterization

Aliquots of 1 mL of inoculum of the strains *P. anomala* CE009 and *P. membranaefaciens* CE015 were transferred to flasks containing 5 mL of yeast malt broth (YMB) and incubated at 18, 21, 24, 27, 31, 33, 36, 39, 42 and 45°C for 48 h. The microbial growth at each temperature was measured by OD at a wavelength of 600 nm after 24 and 48 h. The effect of pH on the growth was evaluated by adjusting the pH in YMB by adding sterile 1 M HCl or 1 M NaOH to pH values of 2, 3, 4, 5, 6, 7, 8, 9 10, 11 and 12. Aliquots of 1 mL of inoculum of the strains *P. anomala* CE009 and *P. membranaefaciens* CE015 were transferred to flasks containing 5 mL of YMB at different pH values. The cultures were incubated at 25°C for 48 h. The microbial growth at each pH was measured by OD at a wavelength of 600 nm at 24 and 48 h.

The influence of salinity on the growth was evaluated by inoculating of 1 mL of each yeast strain in 5 mL of YMB containing 2, 4, 6, 8, 9, 10, 11, 12, 13, 14, 15 and 16% of NaCl (w/v). The media were then incubated at 25°C for 48 h. The microbial growth was measured by OD at 600 nm wavelength after 24 and 48 h.

Ability to grow on petroleum derived hydrocarbons

A mineral salt medium containing in (g L^{-1}): $MgSO_4.7H_2O$ (0.2); $CaCl_2$ (0.02); K_2HPO_4 (13.94); KH_2PO_4 (6.0) and $(NH_4)_2SO_4$ (4.0) was prepared and sterilized at 110°C for 10 min. Afterwards, 0.1%

(v/v) of a micronutrient solution previously sterilized by filtration (0.22 µm, Millipore, USA), was added. The composition of the micronutrient solution per liter was as follows: EDTA (2.5 g), $ZnSO_4.7H_2O$ (10.95 g), $FeSO_4.7H_2O$ (5.0 g), $MnSO_4.H_2O$ (1.54 g), $CuSO.5H_2O$ (0.392 g), Co $(NO_3)2.6H_2O$ (0.25 g), $Na_2B_4O_7.10H_2O$ (0.177 g) (Bushnell and Haas, 1941). This solution was acidified with a few drops of concentrated sulfuric acid, in order to prevent precipitation (Sousa et al., 2012). Finally, substrates n-hexadecane, kerosene and diesel oil, sterilized in a membrane filter (0.22 µm, Millipore), were added aseptically to attain final concentrations of 2% (v/v). Triplicate Erlenmeyer flasks of 250 mL with 50 mL of hexadecane salt medium (HSM), kerosene salt medium (KSM) and oil diesel salt medium (ODSM) were inoculated separately with 1 ml of *P. anomala* CE009 and *P. membranaefaciens* CE015 and incubated at 25°C with shaking speed of 150 rpm for a period of 4 days. To monitor microbial growth, OD of the cultures was measured at 600 nm at intervals of 1 day during 4 days.

Production of biosurfactant

The same procedure described above for the preparation of HSM, KSM and ODSM was applied to prepare glycerol salt medium (GLYSM) and glucose salt medium (GSM) which also were inoculated separately with equal volumes of *P. anomala* CE009 and of *P. membranaefaciens* CE015. The cultures were incubated at 25°C with shaking speed of 150 rpm for 4 days. Afterwards, the cultures were centrifuged at 10,000 g for 15 min, at 4°C and the emulsification index (E_{24}) of the supernatant was measured by adding 2 mL of kerosene and 2 mL of the cell-free broth in a test tube, which was vortexed at high speed for 2 min and allowed to stand for 24 h. After this period, the height of the emulsion layer was measured and the emulsification index (E_{24}) was calculated using Equation (1), according to Desai and Banat (1997):

$$EA_{24\%} = HEL/HS \times 100 \quad (1)$$

Where, HEL is the height of the emulsified layer (cm) and HS is the total height of liquid column (cm). The EA_{24} index was given as percentage of the emulsified layer height (cm) divided by the total height of the liquid column (cm).

Statistical analysis

Statistical analysis was performed by one-way repeated measure ANOVA in association with the post-hoc Tukey test and paired-samples t-test using Prism 5 (GraphPad Software). A $p < 0.05$ was considered significant. All experiments were performed in triplicate with at least two repetitions.

RESULTS

Physiological characterization

Figure 1 shows that the optimum growth conditions were reached at a temperature range of 25-30°C, pH 3.0-4.0 and 2-3% of NaCl for *P. anomala* CE009 and *P. membranaefaciens* CE015. The results also showed that the growth of *Pichia* species decreased under extreme conditions. The growth of the *P. membranaefaciens* CE015 increased until 42°C, while *P. anomala* CE009 increased until 39°C. However, that strain grew up to a NaCl concentration of 15% while *P. membranaefaciens* CE015 only grew up to 10% of that salt, indicating that *P. anomala* CE 009 shows better adaptation to high concentration of NaCl than *P. membranaefaciens* CE015. Both strains grew at extreme pH values of 2 and 12 but, the growth of *Pichia* species differed in their relative sensiti-vity to the factors evaluated. In general, *P. anomala* CE009 showed significantly higher cell concentration ($p<0.05$) than *P. membranaefaciens* CE015 when subjec-ted to the same conditions (Figure 1).

Growth dynamics of two *Pichia* strains on petroleum hydrocarbons (kerosene and diesel oil) and aliphatic hydrocarbon n-hexadecane did not reveal a lag phase and the utilization of hydrocarbon substrates was shown by increase in the number of cells (Figure 2). Hexadecane was used similarly for growth of both *P. anomala* CE009 and *P. membranaefaciens* CE015. The results of kerosene and diesel oil utilization varied significantly between the strains ($p<0.05$). Kerosene was more efficiently used by *P. membranaefaciens* CE015 in comparison to *P. anomala* CE009, which was more efficient in using diesel oil as the carbon and energy source.

Tests carried out with the *P. anomala* CE009 and *P. membranaefaciens* CE015 using kerosene, diesel oil and hexadecane as substrate showed absence of emulsifying activity or unstable emulsification. However, the utilization of the glucose and glycerol by two *Pichia* strains was accompanied by an increase in cell density, with concomitant surfactant activity. The growth dynamics of *P. anomala* CE009 did not reveal a lag phase and the biosurfactant production started at the exponential growth phase (Figure 3A). The growth dynamics of *P. membranaefaciens* CE015 revealed a slight lag phase, but biosurfactant production was not delayed (Figure 3B). For *P. anomala* CE009, the emulsification indexes (E_{24}) ranged from 48 to 58% on glucose and from 39 to 63% on glycerol (Figure 3A). For *P. membranaefaciens* CE015, these ranges were from 38 to 51% on glucose and 40 to 58% on glycerol (Figure 3B). Thus, the highest emulsification indexes for *P. anomala* CE009 were 58 and 63% on glucose and glycerol, respectively, and 51 and 58% for *P. membranaefaciens* CE015 on the same substrates. These values were detected at the end of the exponential growth phase (t = 2 days) and from the fourth day onward a gradual decrease in the two isolates population was observed (Figure 3A and B). The emulsification indexes were higher with glycerol than with glucose for both yeast strains and the emulsification indexes of *P. anomala* CE009 were higher than those of *P. membranaefaciens* CE015 on both substrates.

DISCUSSION

The growth of *P. anomala* CE009 and *P. membranaefaciens* CE015 under extreme values of pH (2 and 12), salinity (2 and 15%) and temperature (18 and 42°C) showed the wide spectrum of plasticity of these strains to environmental conditions. From an ecological point of view, this plasticity can be a tool for survival under the environmental conditions prevalent in the cashew nut processing and oil refinery effluents.

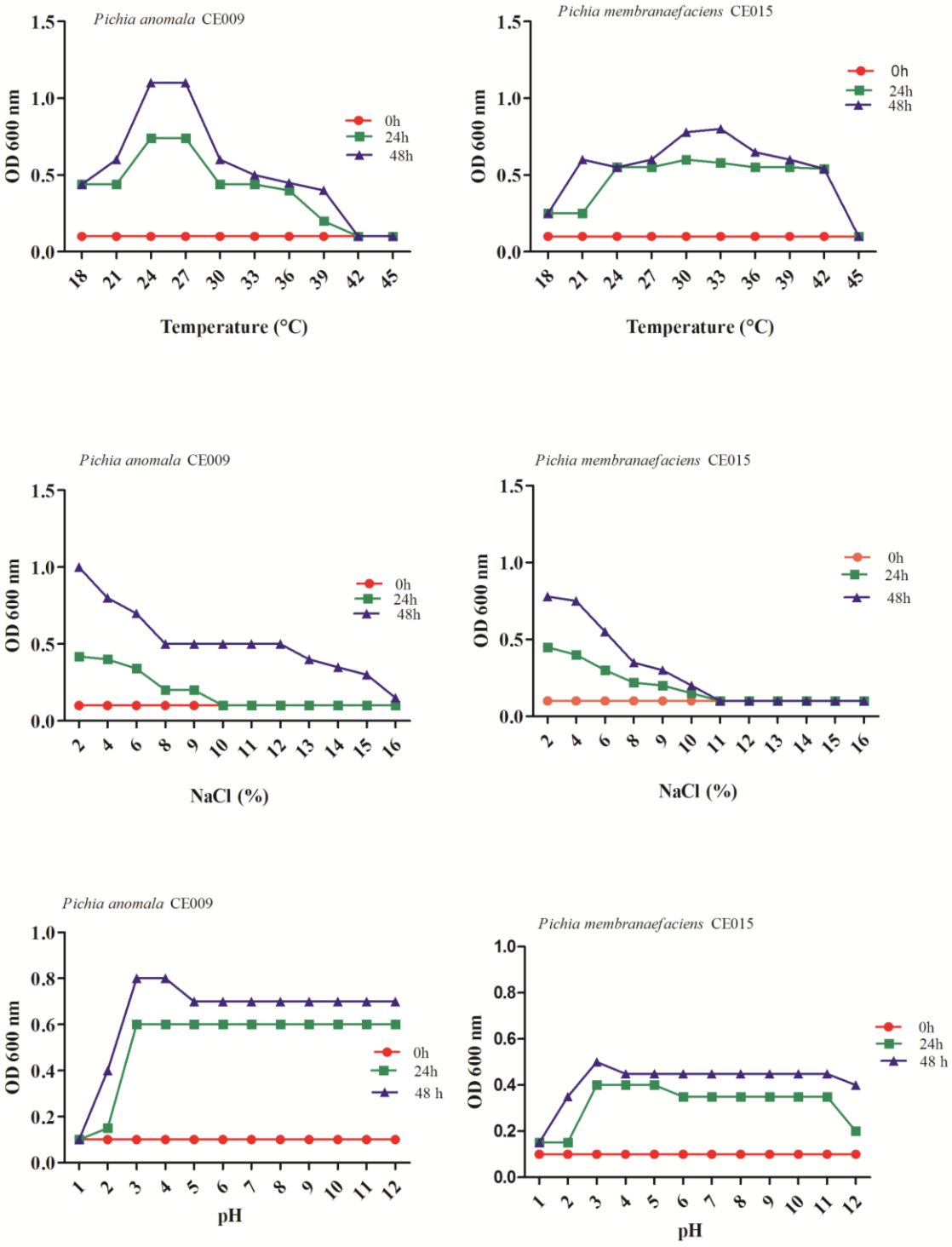

Figure 1. Effects of temperature salinity and pH on growth of *P. anomala* CE009 and *P. membranaefaciens* CE015 in malt broth medium at 0, 24 and 48 h. Each point represents the average value obtained with three independent experiments.

P. anomala CE009 and *P. membranaefaciens* CE015 were categorized as thermotolerant, on the basis of their growth below 20°C, and up to high temperatures, such as 37-48°C (Arthur and Watson, 1976; Limtong et al., 2005). Thermotolerant yeasts have the advantage that they can be cultured under conditions where other microorganisms cannot grow, which reduces the risk of contamination. Thermotolerant enzymes made by yeasts may possess

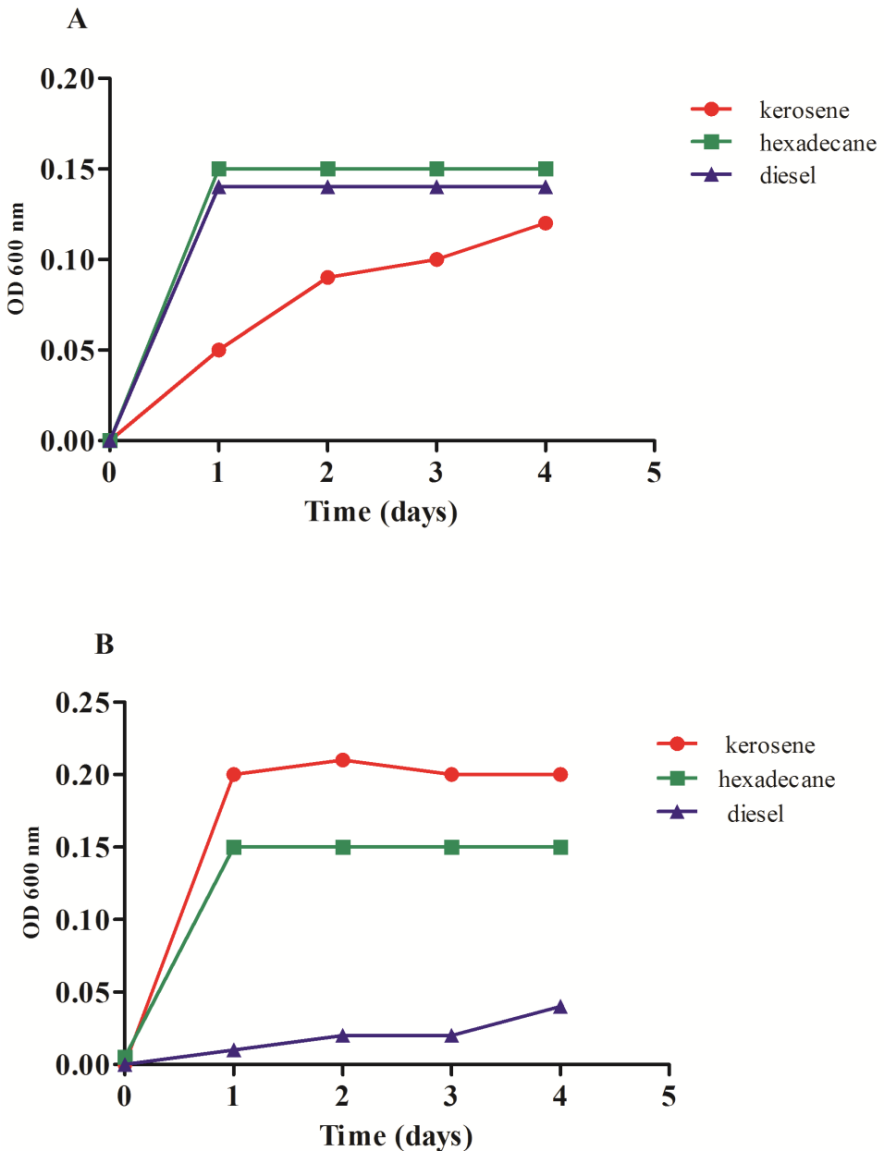

Figure 2. Growth profiles of *P. anomala* CE009 (A) and *P. membranaefaciens* CE015 (B) in mineral salts medium containing 2% of kerosene, diesel oil and hexadecane as the carbon and energy sources. Incubation was done at 26°C with stirring at 150 rpm. Each point represents the average value obtained with three independent experiments.

special advantages because of their eukaryotic nature, when compared with enzymes from thermotolerant bacteria or archaea (Takashima et al., 2009).

In this work, *P. anomala* CE009 and *P. membranaefaciens* CE015 were able to grow in salt concentrations of 10-15% and were classified as halotolerant. High salinity poses osmotic stress and specific ion toxicity for yeast cells (Ren et al., 2012) and salt-tolerance is a strategy of cellular osmotic adaptations at the physiological and molecular level to combat fluctuating salinity (Plemenitaš et al., 2008). Jadhav et al. (2010) emphasized that halotolerant microorganisms are known to be potential sources of extracellular enzymes with novel properties, useful for diverse industrial applications.

Yeasts are usually acid tolerant and grow at pH ≤ 4.0 (Gross and Robbins, 2000), but according to Zvyagilskaya and Persson (2004), pH 10.0 is the upper pH limit for yeast growth and alkalization of the external environment represents a stress situation for most yeast strains (Serrano et al., 2006). Thus, the high alkali tolerance exhibited by *P. anomala* CE009 and *P. membranaefaciens* CE015 makes these *Pichia* strains promising for applications under extreme alkaline environmental conditions.

P. anomala CE009 showed higher cell concentration than *P. membranaefaciens* CE015 under adverse conditions of temperature, pH and salinity, suggesting the better potential of this strain for bioremediation applications in extreme environments.

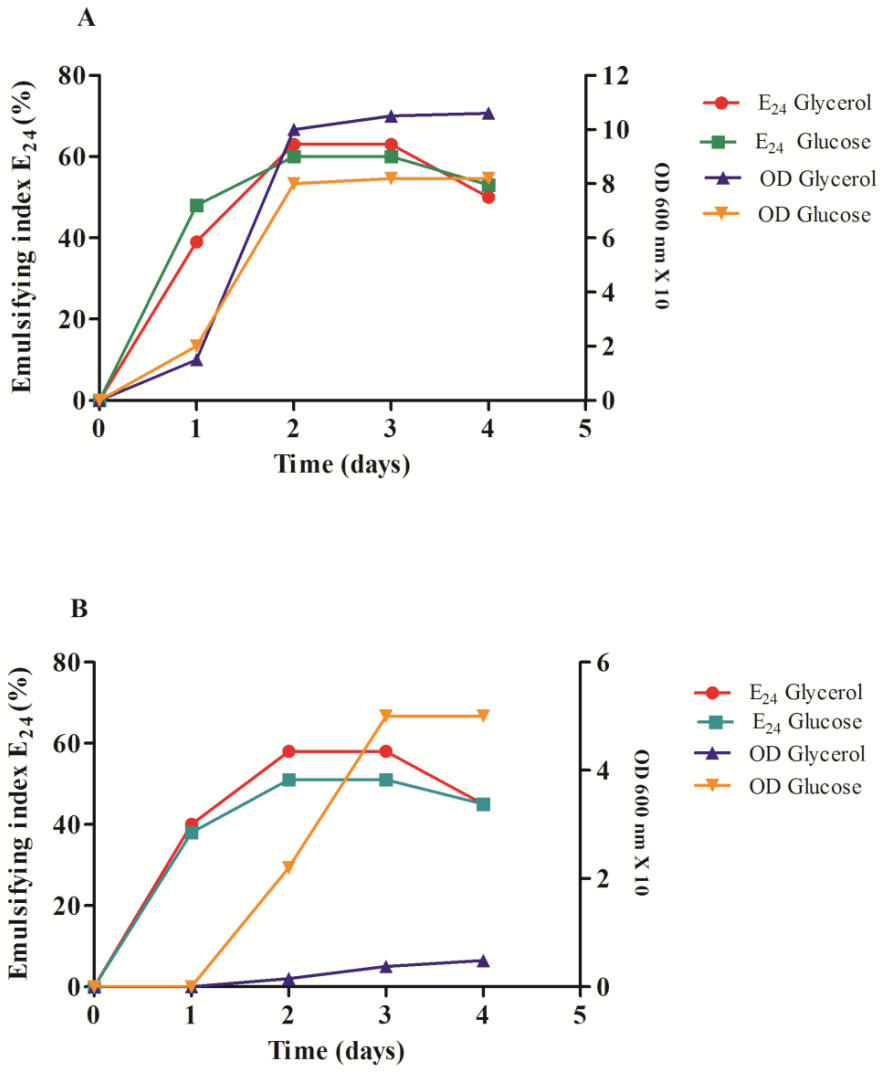

Figure 3. Population dynamics (OD) and emulsification index (E24) during cultivation of yeasts strains *P. anomala* CE009 (A) and *P. membranaefaciens* CE015 (B) on glycerol and glucose. Incubation was done at 26°C with shaking at 150 rpm. Glycerol and glucose were supplied at 2% (v/v). Values are averages of three replicate determinations.

The growth on hexadecane, kerosene and diesel oil indicated the ability of *P. anomala* CE009 and *P. membranefaciens* CE015 for utilization of these petroleum hydrocarbons as carbon and energy source. The absence of lag phase in growth curves of *P. anomala* CE009 and *P. membranefaciens* CE015 was suggestive of the pre-adaptation of these strains to hydrocarbon substrates. For *P. membranaefaciens* CE015 that originated from oil refinery effluent, this result was expected but for *P. anomala* CE009, which was isolated from a cashew nut processing plant effluent, it was surprising. The improvement of the cashew nut is one of the industrial activities that have larger importance, principally economic in States Northeast of the Brazilian. In view of that intense productive activity, those industries generates lots of liquid effluents, whose characteristics are not still well known due to the small amount of scientific works accomplished in the area (Pimentel et al., 2009). However, Rajeswari et al. (2011) reported that the cashew nut shell liquid (CNSL), a phenolic oily liquid is the most abundant by-product of the cashew nut processing which may explain the behavior of *P. anomala* CE009 front to hydrophobic hydrocarbons evaluated in this study.

The maximum value of emulsification index registered was 63% from *P. anomala* CE009 cultivated in glycerol over a period of 2 days. Fontes et al. (2012) reported that *Yarrowia lipolytica* was able to grow and produce biosurfactant on crude glycerol, achieving maximum emulsification index of 70.2%. The fact that biosurfactant production has been higher in the medium containing crude

glycerol than in pure glycerol can be attributed the presence of fatty acids (Fontes et al., 2012), although Yang et al. (2012) have discussed that pollutants in crude glycerol, can inhibit cell growth and result in lower product yields (when compared with commercial glycerol under the same culture conditions). Chandran and Das (2010) showed that emulsification index of the Tween 80 was 67% and the present study showed that the value to *P. anomala* CE009 was quite higher than the chemical commercial surfactant.

The profile of growth curves of *P. anomala* CE009 and *P. membranaefaciens* CE015 on hydrophilic substrates (Figure 3), confirmed that glycerol usually serves as a substitute for glucose (Bognolo, 1999). To Ilori et al. (2008), the exact reasons why some microorganisms produce biosurfactants are still not clear but according Salihu et al. (2009), the carbon source is very important in the production of these substances and includes carbohydrates, hydrocarbons and vegetable oils. To Abasi et al. (2012), the microorganisms differ in their capacity to produce biosurfactants, some use as substrates only carbohydrates, others only hydrocarbons, and still others consume several substrates, in combination or separately. In this work, while the hydrophobic substrates were inefficient for biosurfactant production the hydrophilic substrates (glucose and glycerol) were used for this objective by *P. anomala* CE009 and *P. membranaefacien*s CE015. These observations are in accordance with data in the literature on the synthesis of biosurfactants by yeasts strains from hydrophilic substrates such as glucose (Dziegielewska and Adamczak, 2013) and sugarcane molasses (Takahashi et al., 2011). Wu et al. (2008) reported similar results for *Pseudomonas aeruginosa* EM1, while, Queiroga et al. (2003) showed that *Bacillus subtilis* C9 produced high yields of biosurfactant using a soluble carbohydrate substrate, while a hydrocarbon substrate inhibited the production of this substance. In contrast, Nitschke et al. (2005) described that hydrophilic carbon sources such as glycerol present lower yield of biosurfactants as compared to hydrophobic sources such as soybean oil.

Although, most biosurfactants are considered secondary metabolites (Singh and Cameotra, 2004), in this study, the emulsifying index values indicate that the biosurfactant biosynthesis occurred predominantly during the exponential growth phase, suggesting that the biosurfactant is produced as primary metabolite accompanying cellular biomass formation (Cunha et al., 2004). For *P. anomala* CE009 and *P. membranaefaciens* CE015 the biosurfactant production ceased when growth stopped and population density started decreasing (Figure 3) probably due to the production of secondary metabolites that could interfere with emulsion formation. Khopade et al. (2012a) also reported that a biomulsifier synthesized by a strain of *Bacillus* sp. was produced as a primary metabolite accompanying a cellular biomass formation. In contrast, Amaral et al. (2006) and Accorsini et al. (2012) reported that the most biosurfactants are usually produced when the cultures reach the stationary phase of growth.

This affirmation is corroborated by Ilori et al. (2008) which showed that biosurfactant synthesis by the *Saccharomyces cerevisiae* and *Candida albicans* was maximal for cells in stationary/death phase and in accordance with previously reported data by Rodrigues et al. (2006) on kinetic study of biosurfactant production by *Lactobacillus* strains. It was found that the best biosurfactant-producing *Pichia* species was *P. anomala* CE009 with an E_{24} of 63%. *P. anomala* PY1 was described by Thaniyavarn et al. (2008) as biosurfactant producer by using soybean oil as carbon source. Except for this report, other *Pichia* species such as *P. membranaefaciens* has not been reported previously for biosurfactant production which highlights the innovative aspect of this work.

On the other hand, according to Dobson et al. (2012), although the current price of glucose is comparable to that of crude glycerol (US $0.21-0.23/lb), strong price fluctuation has been observed in the last 15 years, with prices reaching US $0.40/lb at the beginning of 2010. In addition, recent surge in biodiesel production has led to increased accumulation of glycerol as byproduct of this industry. As consequence, the price of crude glycerol is continuously decreasing and a negative value will be attributed to crude glycerol in the future, which will increase the interest to use it as a biological feedstock for the production of economically value-added products, as the biosurfactants (Coombs, 2007; Pagliaro et al., 2009), but few publications present the possibility to utilize waste glycerol for biosurfactant synthesis (Morita et al., 2007). Although this study has utilized glycerol comercial the results indicate the potential of *P. anomala* CE009 and *P. membranaefaciens* CE015, particularly the first strain, for biosurfactant production from glycerol.

Conclusion

P. anomala CE009 and *P. membranaefaciens* CE015 represent a potential, still unexploited, for studies on biotransformation of toxic pollutants and biosurfactant production on co-substrates such as sugar and glycerol.

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REFERENCES

Abbasi H, Hamedi MM, Lotfabad TB, Zahiri HS, Sharafi H, Masoomi F, Moosavi-Movahedi AA, Ortiz A, Amanlou M, Noghabi KA (2012). Biosurfactant-producing bacterium, *Pseudomonas aeruginosa* MA01 isolated from spoiled apples: physicochemical and structural characteristics of isolated biosurfactant. J. Biosci. Bioeng. 113:211-219.

Abouseoud M, Maachi R, Amrane A, Boudergua S, Nabi A (2008). Evaluation of different carbon and nitrogen sources in production of biosurfactant by *Pseudomonas fluorescens*. Desalination. 223:143-151.

Accorsini FR, Mutton MJR, Lemos EGM, Benincasa M (2012). Biosurfactants production by yeasts using soybean oil and glycerol as low cost substrate. Braz J Microbiol. 43:116-125.

Amaral PFF, da Silva JM, Lehocky M, Barros-Timmons AMV, Coelho MAZ, Marrucho IM, Coutinho JAP (2006). Production and characterization of a bioemulsifier from *Yarrowia lipolytica*. Process Biochem. 41:1894-1898.

Arthur H, Watson K (1976). Thermal adaptation in yeast: growth temperatures, membrane lipid and cytochrome composition of psychrophilic, mesophilic, and thermophilic yeasts. J. Bacteriol. 128: 56-68.

Banat IM, Franzetti A, Gandolfi I, Bestetti G, Martinotti MG, Fracchia L, Smyth TJ, Marchant R (2010). Microbial biosurfactants production, applications and future potential. Appl Microbiol Biotechnol. 87:427-444.

Batista RM, Rufino RD, Luna JM, de Souza JEG, Sarubbo L (2010). Effect of medium components on the production of a biosurfactant from *Candida tropicalis* applied to the removal of hydrophobic contaminants in soil. Water Environ Res. 82: 418-425.

Bognolo F (1999). Biosurfactants as emulsifying agents for hydrocarbons. Colloids Surf A. 152:41-52.

Burgos-Díaz C, Pons R, Espuny MJ, Aranda FJ, Teruel, JA, Manresa A, Ortiz A, Marqués AM (2013). The production and physicochemical properties of a biosurfactant mixture obtained from *Sphingobacterium detergens*. J. Colloid Interface Sci. 394: 368-379.

Bushnell DL, Haas HF (1941). The utilization of certain hydrocarbons by microorganisms. J Bacteriol. 41:653-673.

Chandran P, Das N (2010). Biosurfactant production and diesel oil degradation by yeast species *Trichosporon asahii* isolated from petroleum hydrocarbon contaminated soil. Int J Eng Sci Technol. 2:6942-6953.

Chénier MR, Beaumier D, Roy R, Driscoll BT, Lawrence JR, Greer CW. (2003). Impact of seasonal variations and nutrient input on nitrogen cycling and degradation of hexadecane by replicated river biofilms. Appl. Environ. Microbiol. 69:5170-5177.

Chung BK, Lakshmanan M, Klement M, Ching CB, Lee DY (2013). Metabolic reconstruction and flux analysis of industrial *Pichia* yeasts. Appl Microbiol Biotechnol. 97:1865-1873.

Coimbra CD, Rufino RD, Luna JM, Sarubbo LA (2009). Studies of the cell surface properties of *Candida* species and relation with the production of biosurfactants for environmental applications. Curr Microbiol. 58:245-249.

Coombs A (2007). Glycerin bioprocessing goes green. Nat Biotechnol. 25: 953-954.

Cunha CD, Rosario M, Rosado AS, Leite SGF (2004). *Serratia* sp. SVGG16: A promising biosurfactant producer isolated from tropical soil during growth with ethanol-blended gasoline. Process. Biochem. 39: 2277-2282.

Das P, Mukherjee S, Sen R (2009). Substrate dependent production of extracellular biosurfactant by a marine bacterium. Bioresour Technol. 100:1015-1019.

Daverey A, Pakshirajan K (2010). Sophorolipids from *Candida bombicola* using mixed hydrophilic substrates: Production, purification and characterization. Colloids Surf, B. 79: 246-256.

Desai JD, Banat IM (1997). Microbial production of surfactants and their commercial potential. Microbiol Mol Rev. 61: 47-64.

Dobson R, Gray V, Rumbold K (2012). Microbial utilization of crude glycerol for the production of value-added products. J Ind Microbiol Biotechnol. 39: 217-226.

Dziegielewska E, Adamczak M, (2013). Evaluation of waste products in the synthesis of surfactants by yeasts. Chem Pap. 67: 1113-1122.

Eddouaouda K, Mnif S, Badis A, Yones SB, Cherif S, Ferhat, S, Mhiri N, Chamkha M, Sayadi S (2012). Characterization of a novel biosurfactant produced by Staphylococcus sp. strain 1E with potential application on hydrocarbon bioremediation. Journal of Basic Microbiology. 58:408-418.

Fontes GC, Amaral PFF, Coelho MAS (2008). Biosurfactants production by yeasts. Quím Nova. 31: 2091-2099.

Fontes GC, Ramos NM, Amaral PFF, Nele M, Coelho AZ (2012). Renewable resources for biosurfactant production by *Yarrowia lipolytica*. Braz. J. Chem. Eng. 29: 483-493.

Gross S, Robbins EI (2000). Acidophilic and acid-tolerant fungi and yeasts. Hydrobiologia. 433: 91-109.

Ilori MO, Adebusoye SA, Ojo AC (2008). Isolation and characterization of hydrocarbon-degrading and biosurfactant-producing yeast strains obtained from a polluted lagoon water. World J Microbiol Biotechnol. 24: 2539-2545.

Jadhav GG, Salunkhe DS, Nerkar DP, Bhadekar RK (2010). Isolation and characterization of salt-tolerant nitrogen-fixing microorganisms from food. EurAsia J BioSci. 4: 33-40.

Khopade A, Biao R, Liu X, Mahadik K, Zhang L, Kokare C (2012a). Production and stability studies of the biosurfactant isolated from marine *Nocardiopsis* sp. B4, Desalination. 85: 198-204.

Khopade A, Ren B, Liu XY, Mahadik K, Zhang L, Kokare, C (2012b). Production and characterization of biosurfactant from marine *Streptomyces* species B3. J. Colloid Interface Sci. 367: 311-318.

Kurtzman PC, Fell JW (1998). The yeasts: a taxonomic study. 4th ed. Amsterdam: (eds.) Elsevier, 1055 p.

Limtong S, Srisuk N, Yongmanitchai W, Yurimoto H, Nakase T, Kato N (2005). *Pichia thermomethanolica* sp. nov., a novel thermotolerant, methylotrophic yeast isolated in Thailand. Int J Syst Evol Micr. 55: 2225-2229.

Luna JM, Sarubbo L, Campos-Takaki GMA (2009). New Biosurfactant produced by *Candida glabrata* UCP 1002: Characteristics of stability and application in oil recovery. Braz Arch Biol Technol. 52: 785-793.

Luna JM, Rufino RD, Sarubbo LA, Rodrigues LRM, Teixeira JAC, Campos-Takaki GM (2011). Evaluation antimicrobial and antiadhesive properties of the biosurfactant lunasan produced by *Candida sphaerica* UCP 0995. Curr Microbiol. 62: 1527-1534.

Luna JM, Rufino RD, Campos-Takaki GM, Sarubbo LA (2012). Properties of the biosurfactant produced by *Candida sphaerica* cultivated in low-cost substrates. Chem Eng Trans. 7: 67-72.

Morita T, Konishi M, Fukuoka T, Imura T, Kitamoto D (2007). Microbial conversion of glycerol into glycolipid biosurfactants, mannosylerythritol lipids, by a basidiomycete yeast, *Pseudozyma antarctica* JCM 10317T. J Biosci Bioeng. 104: 78-81.

Morita T, Konishi M, Fukuoka T, Imura T, Kitamoto D (2008). Production of glycolipid biosurfactants, mannosylerythritol lipids, by *Pseudozyma siamensis* CBS 9960 and their interfacial properties. J Biosci Bioeng. 105: 493-502.

Nitschke M, Costa SG, Haddad R, Goncalves LA, Eberlin MN, Contiero J (2005). Oil wastes as unconventional substrates for rhamnolipid biosurfactant production by *Pseudomonas aeruginosa* LBI. Biotechnol Prog. 21: 1562-1566.

Pagliaro M, Ciriminna R, Kimura H, Rossi M, Della Pina C (2009). Recent advances in the conversion of bioglycerol into value-added products. Eur J Lipid Sci Technol. 111: 788-799.

Pansiripat S, Pornsunthorntawee O, Rujiravanita R, Kitiyanana B, Somboonthanate P, Chavadeja S (2010). Biosurfactant production by *Pseudomonas aeruginosa* SP4 using sequencing batch reactors: Effect of oil-to-glucose ratio. Biochem Eng J Bioch Eng. 49: 185-191.

Pimentel MF, Lima DP, Martins LR, Beatriz A, Santaella ST, Lotufo LVC (2009). Ecotoxicological analysis of cashew nut industry effluents, specifically two of its major phenolic components, cardol and cardanol. PanamJas. 4: 363-368.

Plemenitaš A, Vaupotič T, Lenassi M, Kogej T, Gunde-Cimerman N (2008) Adaptation of extremely halotolerant black yeast *Hortaea werneckii* to increased osmolarity: a molecular perspective at a glance. Stud Mycol. 61: 67-75.

Queiroga CL, Nascimento LR, Serra GE (2003). Evaluation of paraffins biodegradation and biosurfactant production by *Bacillus subtilis* in the present of crude oil. Braz J Microbiol. 34: 321-324.

Rajeswari T, Padmapriya B, Teesha K, Kavitha Kumari K (2011). Degradation of cashew nut shell liquid by *Pseudomonas* sp isolated from soil. International J Microbiol Res. 2: 172-175.

Ren H, Wang X, Liu D, Wang B (2012). A glimpse of the yeast *Saccharomyces cerevisiae* responses to NaCl stress. Afr J Microbiol Res. 6: 713-718.

Rodrigues LR, Moldes A, Teixeira JA, Oliveira R (2006). Kinetic study of fermentative biosurfactant production by *Lactobacillus* strains. Biochem Engin J. 28:109-116.

Rufino RD, Luna JM, Campos-Takaki GM, SRM, Sarubbo LA (2012). Application of the biosurfactant produced by *Candida lipolytica* in the remediation of heavy metals. Chem. Eng. Trans. 27:61-66.
Salihu A, Abdulkadir I, Almustapha MN (2009). An investigation for potential development on biosurfactants. Biotechnol. Mol. Biol. Rev. 3: 111-117.
Schoefs O, Perrier M, Samson R (2004). Estimation of contaminant depletion in unsaturated soils using a reduced-order biodegradation model and carbon dioxide measurement. Appl. Microbiol. Biotechnol. 64:53-61.
Serrano R, Martin H, Casamayor A, Arino J (2006). Signaling alkaline pH stress in the yeast *Saccharomyces cerevisiae* through the Wsc1 cell surface sensor and the Slt2 MAPK Pathway. J. Biol. Chem. 281: 39785–39795.
Silva GP, Mack M, Contiero J (2009). Glycerol: A promising and abundant carbon source for industrial microbiology. Biotechnol Adv. 27: 30-39.
Singh P, Cameotra S (2004). Potential applications of microbial surfactants in biomedical sciences. Trends Biotechnol. 22: 142-146.
Sousa M, Melo VMM, Rodrigues S, Sant'ana HB, Gonçalves LRB (2012). Screening of biosurfactant-producing *Bacillus* strains using glycerol from the biodiesel synthesis as main carbon source. Bioprocess Biosyst. Eng. 35: 897-906.
Takashima M, Sugita T, Toriumi Y, Nakase T (2009). *Cryptococcus tepidarius* sp. nov., a thermotolerant yeast species isolated from a stream from a hotspring area in Japan. Int. J. Syst. Evol. Micr. 59: 181-185.
Takahashi M, Morita T, Wada K, Hirose N, Fukuoka T, Imura T, Kitamoto D (2011). Production of sophorolipid glycolipid biosurfactants from sugarcane molasses using *Starmerella bombicola* NBRC 10243. J. Oleo Sci. 60: 267-273.
Thaniyavarn J, Chianguthai T, Sangvanich P, Roogsawang N, Washio K, Morikawa M, Thaiyavarn S (2008). Production of sophorolipid biosurfactant by *Pichia anomala*. Biosci. Biotechnol. Biochem. 72: 2061-2068.
Walker GM (2011). *Pichia anomala*: cell physiology and biotechnology relative to other yeasts. Antonie Van Leeuwenhoek. 99: 25-34.
Wu JY, Yeh KL, Lu WB, Lin CL, Chang JS (2008). Rhamnolipid production with indigenous *Pseudomonas aeruginosa* EM1 isolated from oil-contaminated site. Bioresour. Technol. 99: 1157-1164.
Yang F, Hanna MA, Sun R (2012). Value-added uses for crude glycerol-a byproduct of biodiesel production. Biotechnol Biofuels. 5: 1-10.
Yin H, Qiang Y, Jia Y, Ye J, Peng H, Quri H, Zhang N, He B. (2009). Characteristics of biosurfactant produced by *Pseudomonas aeruginosa* S6 isolated from oil-containing wastewater. Proc. Biochem. 44:302-308.
Zvyagilskaya RA, Persson BL (2004). A new alkalitolerant *Yarrowia lipolytica* yeast strain is a promising model for dissecting properties and regulation of Na+-dependent phosphate transport systems. Biochemistry. 69: 1310-1317.

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Evaluation of an alternative chromogenic method for the detection and enumeration of enterococci in waters

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A new chromogenic method for the detection and enumeration of enterococci in water was evaluated using water samples from different sources, specifically, industrial, potable and recreational waters, and compared with the standard methodology for water quality ISO 7899-2. Several statistical parameters, such as relative accuracy, selectivity, efficiency, limit of detection, limit of quantification, inclusivity and exclusivity were determined according to the main criteria provided in ISO 16140 and ISO 17994. An orthogonal regression analysis demonstrated that the developed alternative method performs similar to ISO 7899. The chromogenic medium allowed easy recognition of enterococci by the appearance of pink-colored colonies. Importantly, the method can be executed in only 24 h, showing a similar selectivity (-0.05), a lower limit of quantification (4.08 cfu/100 ml), and a higher efficiency (98%) than the reference method (-0.04; 5.50 cfu/100 ml and 97%, respectively). The results obtained support the use of the alternative method for the detection and enumeration of enterococci to assess water quality from several sources.

Keywords: Enterococci, chromogenic method, water quality.

INTRODUCTION

The quality of water is routinely monitored to protect human from fecal contamination, using indicator bacteria, such as total coliforms, fecal coliforms, *Escherichia coli* and enterococci. The bacterial genus *Enterococcus* is traditionally considered as hygiene indicator due to its ability to persist longer in the environment than other bacteria. Its detection methods are essential elements of safe drinking water regulations worldwide (Frahm and Obst, 2003). Moreover, several studies suggest that bacteria of this genus can be more effective indicators than *E. coli* and fecal coliforms to assess sanitary water quality (Pepe et al., 2011).

Two procedures are currently used for the detection of such microorganisms in water, the most probable number (MPN) method and the membrane filtration (MF) technique (Volterra et al., 1985). Both include conventional culture media in which the identification of the microorganism is based on its physiological characteristics taking a long time to provide results (Domig et al., 2003). In the last few years, several chromogenic and fluorogenic culture media have been developed as alternative methods for such purposes. These media contain

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Table 1. Composition of m-CromoCen ENT.

Composition	g/l
Tryptone	2·0
Yeast extract	1·0
Peptone P (pancreatic beef heart peptone)	1·0
Peptone Z (papainic beef heart peptone)	1·0
Meat extract	3·0
Rose-glu	0·075
Thallium acetate	0·475
Xylose	10·0
Dipotassium phosphate	5·0
Dihydrogen potassium phosphate	1·5
Bromocresol purple	0·01
Agar	13·0
Total	38·06

multiple substrates that allow bacteria to form colored colonies depending on their enzyme activity, precluding the need of subsequent cultivation steps and additional biochemical tests to establish the identity of the isolated microorganisms. Furthermore, the chromogenic and fluorogenic culture media also enhance the discrimination of different species in mixed cultures, which provides better sensitivity when compared with conventional culture media (Orenga et al., 2009).

In addition, enterococci are considered important nosocomial pathogens (Hajia et al., 2012). A variety of chromogenic media have been developed for the detection of enterococci in clinical samples (Gander et al., 2013).

The aim of this study was to assess the suitability of a new alternative chromogenic method for the detection and enumeration of enterococci in water samples by the MF technique, in comparison with the ISO 7899:2 method (ISO 7899:2, 2000).

MATERIALS AND METHODS

Samples

A total of 135 water samples from different sources were examined with the MF technique from March 2007 to March 2012: 59 industrial water samples (chlorinated), 43 drinking water (chlorinated and coming from an aqueduct), and 33 recreational waters (swimming pools). All samples were collected using sterile bottles, chilled for transportation, and processed within 6 h after collection.

Of these samples, 94 were spiked with *Enterococcus faecalis* ATCC 29212 strain, to achieve five different levels of contamination (absence level, 1-10, 11-30, 31-100 and more than 100 cfu/100 ml). Before testing, residual chlorine was measured by a colorimetric method using Aquaquant® (Darmstadt, Germany), and inactivated by adding a sterile solution of sodium thiosulphate solution at 18% (5 ml/l) (w/v).

Culture media

The m-CromoCen ENT chromogenic medium (BioCen, Cuba) was used as an alternative method, Slanetz-Bartley (SB) agar (Oxoid, Ltd, Basingstoke, UK) was used as a presumptive reference medium, and bile-esculin azide (BAA) agar (Merck, Darmstadt, Germany) served as confirmatory reference medium. All the media were prepared according to manufacturer's instructions. The composition of the m-CromoCen ENT is shown in Table 1.

Microbiological analysis

A volume of 100 ml of each water sample was filtered through a sterile nitrocellulose membrane (47-mm diameter and 0.45-µm pore size, Sartorius, Gottingen, Germany). The filter was rinsed before and after each filtration with 40 ml of a sterile phosphate buffered solution. The membranes were incubated on plates of m-CromoCen ENT medium during 24 h at 35 ± 2°C. Later, all colonies from light to dark pink color were considered typical. This procedure was compared with ISO 7899-2:2000 for water quality (ISO 7899:2, 2000).

After the corresponding incubation time, enterococcal colonies in each culture medium were enumerated. The results were expressed as colony forming units (cfu) per 100 ml.

Biochemical identification

Whenever it was possible, 10 colonies per sample were selected from these media. When the number of colonies per sample was smaller, all colonies were analyzed. The colonies were identified using a set of 19 biochemical tests: catalase, oxidase, motility, growth on sheep blood agar, hemolysis, arginine hydrolysis, growth in 6·5% sodium chloride, growth in 40% bile, growth at 45°C, growth at 9·6 pH, hydrolysis of esculin, Voges-Proskauer test, growth on Chromocult® enterococci (Merck, Germany), fermentation of pyruvate, arabinose, mannitol, sorbitol, lactose and sorbose. The rapid PYR test (hydrolysis of L-pyrrolydonyl-β-naphtylamide, O.B.I.S. PYR, Oxoid LTD, Basingstoke, UK) was performed to confirm doubtful results (Holt et al., 1994; Domig et al., 2003).

Inclusivity and exclusivity tests were performed. For the inclusivity study, 44 strains of target microorganisms were evaluated, including 6 ATCC strains and 38 clinical isolates from hospitals. For the exclusivity assay, 43 strains of non-target microorganisms were tested, mainly Gram-negative bacteria; of them, 26 ATCC strains and clinical isolates. In addition, some representative yeasts strains and Gram-positive bacteria were included in the test. The purity of all clinical isolated microorganisms used in this study was previously confirmed by biochemical tests.

Statistical analysis

The statistical analysis (orthogonal regression), and calculation of the other parameters, such as selectivity (F), efficiency (E), limit of quantification (LOQ) and limit of detection (LOD), were performed according to ISO 16140 (ISO 16140, 2003). Similarly, the ability of the alternative method to measure enterococci in all water matrices was evaluated. Data of enterococci colony counts of both methods were transformed to $\log_{10}$ to obtain a normal distribution of data and facilitate the statistical analysis. Results were analyzed using orthogonal regression to verify the linearity of the relationship between the results obtained with both the m-CromoCen ENT method and the ISO 7899-2:2000 reference. In samples where no growth was observed (absence level), logarithmic transformation was performed by taking the log of value 1; consequently, in the remainder samples, 1 cfu was added to each value of the count

obtained and the sums were subjected to logarithmic transformation. The orthogonal regression analysis was undertaken to assess the relative accuracy between both methods. Three regression equations were independently calculated for each matrix and one for the overall matrices.

To determine the relative limit of detection, the water samples were spiked with *E. faecalis* ATCC 29212 at seven different concentrations and expressed as cfu/100 ml. Six subsamples of each level of contamination were filtered and the growth of enterococci with both methods was reported. The values were calculated by means of a regression analysis, taking into account the number of Petri dishes with growth and the overall number of inoculated plates in each level. To verify the hypothesis that both methods have the same level of detection a 2 × 2 contingency table was created and the exact Fisher test was applied. The analysis was also done according to the Nordval formula (NordVal, 2009).

Determination of LOQ was done using the same procedure used for LOD, but quantifying the number of colonies in each of the six plates of each level. LOQ was calculated for α = 5% and (1-β) = 50% (ISO 16140, 2003).

RESULTS

All strains tested in the inclusivity study exhibited expected typical colony characteristics on both, the alternative and the reference medium with a 100% value. Strains showed pink colonies on m-CromoCen ENT medium; red, maroon or pink colonies on Slanetz-Bartley agar and red or maroon colonies surrounded by a black or brown halo.

In the exclusivity study, most Gram-negative microorganisms and yeasts evaluated on m-CromoCen ENT were inhibited. Some non-target Gram-positive and negative microorganisms grew moderately, showing a different color from that exhibited by enterococci. In the reference method, the characteristic enterococcus colonies or the typical inhibition of non-target microorganisms was observed.

Out of 135 water samples analyzed, 31 showed no microbial growth (absence level) and 102 had enterococci isolated by both methods. Enterococci were isolated by only one method (reference method) in two of all samples. The results of the enumeration of enterococci obtained by the two methods were compared, being enterococci counts, in most of the samples, slightly higher by the alternative method. The range of enterococci counts of all water matrices obtained with alternative and reference methods is shown in Table 2.

Enterococci were isolated by both methods from different sources of natural water, even in samples properly chlorinated. Furthermore, in the artificially spiked samples, several enterococcal species were isolated. In general, most isolates on m-CromoCen ENT corresponded to *E. faecalis* (77·05%), followed by *Enterococcus faecium* (3·85%), and *Enterococcus solitarius* (1·51%). Other *Enterococcus* species were isolated in minor proportion (< 1%).

The chromogenic method showed a selectivity of -0.05 and an efficiency of 98%. The percentage of enterococci that grew on m-CromoCen ENT as non-typical colonies did not exceed 10%. The rates of false-positive and false-negative obtained were 0 and 1.92, respectively.

In all types of water analyzed, the regression analysis for enterococci showed strong correlation between both methods. In all water matrices studied, the intercept was close to zero and the slope value was close to 1, although m-CromoCen ENT gave slightly higher estimates than the reference, demonstrating a high degree of linearity (Figure 1).

The regression slope (b = 1.02) indicates a close relation of the alternative method with regard to the ISO 7899:2 method. The hypothesis verification for the regression showed that the calculated F was lower than the critical F; therefore, there was a linear relation (ISO 16140, 2003).

Concerning LOD, for the five levels studied, critical F was 2.66 and calculated F was 1.36. The relative limit of detection obtained was 0.3 cfu/100 ml. In the exact Fisher test performed to verify the hypothesis, a P = 0·54 indicates that both methods have the same level of detection. The results of the 2 × 2 contingency table, created to verify the hypothesis that both methods have the same level of detection, are shown in Table 3.

The relative detection limit calculated proved that the confidence intervals of the alternative method are equal or are included in those of the reference method. The detection limit attained was 0.75 cfu/100 ml.

The LOQ of the alternative method was 4.08 cfu/100 ml, however the reference method showed a LOQ of 5.48 cfu/100 ml. The chromogenic method studied gave even positive results with as few as 4 cfu/100 ml of enterococci after 24 h.

DISCUSSION

There are many conventional culture methods to detect enterococci in water, but these traditional culture-based methods require a minimum of 24 h incubation period, followed by a confirmation procedure that can take up to several days, which compromises the ability to take the most appropriate and timely action. The need to have rapid detection methods has increased, especially in the water industry and in emergency situations (González et al., 2009).

The alternative method developed to monitor water quality, using enterococci as indicator of fecal pollution, is a chromogenic medium based on the detection of β-D-glucosidase activity present in enterococci (Perry et al., 2007). Several chromogenic and fluorogenic culture media have been described for enterococci detection using the same principle (Manafi, 2000). On m-CromoCen ENT agar, pink colonies allows for easier recognition of enterococci in the different water samples analyzed.

The substrate included in the medium is stable at

Table 2. Range of counts for enterococci by the studied level of water samples obtained with alternative and reference methods.

Level (cfu/100 ml)	Source	Type	Range (cfu/100 ml)		Average		Standard deviation	
			Reference method (48 h)	Alternative method (24 h)	Reference method	Alternative method	Reference method	Alternative method
1-10	Industrial water	Natural	1-9	1-27	3·50	8·50	3·79	12·37
		Spiked	1-10	0-41	4·42	8·75	2·71	11·86
	Drinking water	Natural	1-6	1-11	2·25	4·50	2·50	4·51
		Spiked	8-9	10-13	8·67	11·33	0·58	1·53
	Recreational water	Natural	1-6	0-2	3·33	1·33	2·52	1·15
		Spiked	8-10	6-12	9	9·67	1·00	3·21
11-30	Industrial water	Natural	-	-	-	-	-	-
		Spiked	12-28	13-29	20·90	20·70	5·74	5·72
	Drinking water	Natural	22	30	22	30	-	-
		Spiked	13-29	10-35	23·67	25·50	6·86	9·89
	Recreational water	Natural	22	7	22	7	-	-
		Spiked	14-24	12-17	16·20	15·40	4·38	2·07
31-100	Industrial water	Natural	-	-	-	-	-	-
		Spiked	32-78	25-87	51·00	53·23	14·62	17·56
	Drinking water	Natural	31	124	31	124	-	-
		Spiked	33-86	33-116	59·00	64·45	17·56	26·82
	Recreational water	Natural	-	-	-	-	-	-
		Spiked	39-89	40-108	63·57	68·00	21·56	24·93
> 100	Industrial water	Natural	-	-	-	-	-	-
		Spiked	113-347	122-259	205·5	195·88	82·23	55·20
	Drinking water	Natural	-	-	-	-	-	-
		Spiked	104-263	114-245	156·33	174·00	56·89	48·56
	Recreational water	Natural	-	-	-	-	-	-
		Spiked	112-213	103-208	166·50	165·00	45·94	49·76

115°C for 15 min and thus can be added prior to sterilization, making the preparation of m-CromoCen ENT medium easier and faster. Importantly, with the alternative method only one medium and a rapid test for target bacteria confirmation were necessary. This full procedure requires only 24 h to produce results, in contrast with ISO 7899:2 that takes 48 h and uses two expensive culture media, containing sodium azide, an inhibitor highly harmful to health (Chang and Lamm, 2003; Demircan et al., 2011). Moreover, the chromogenic method can be used

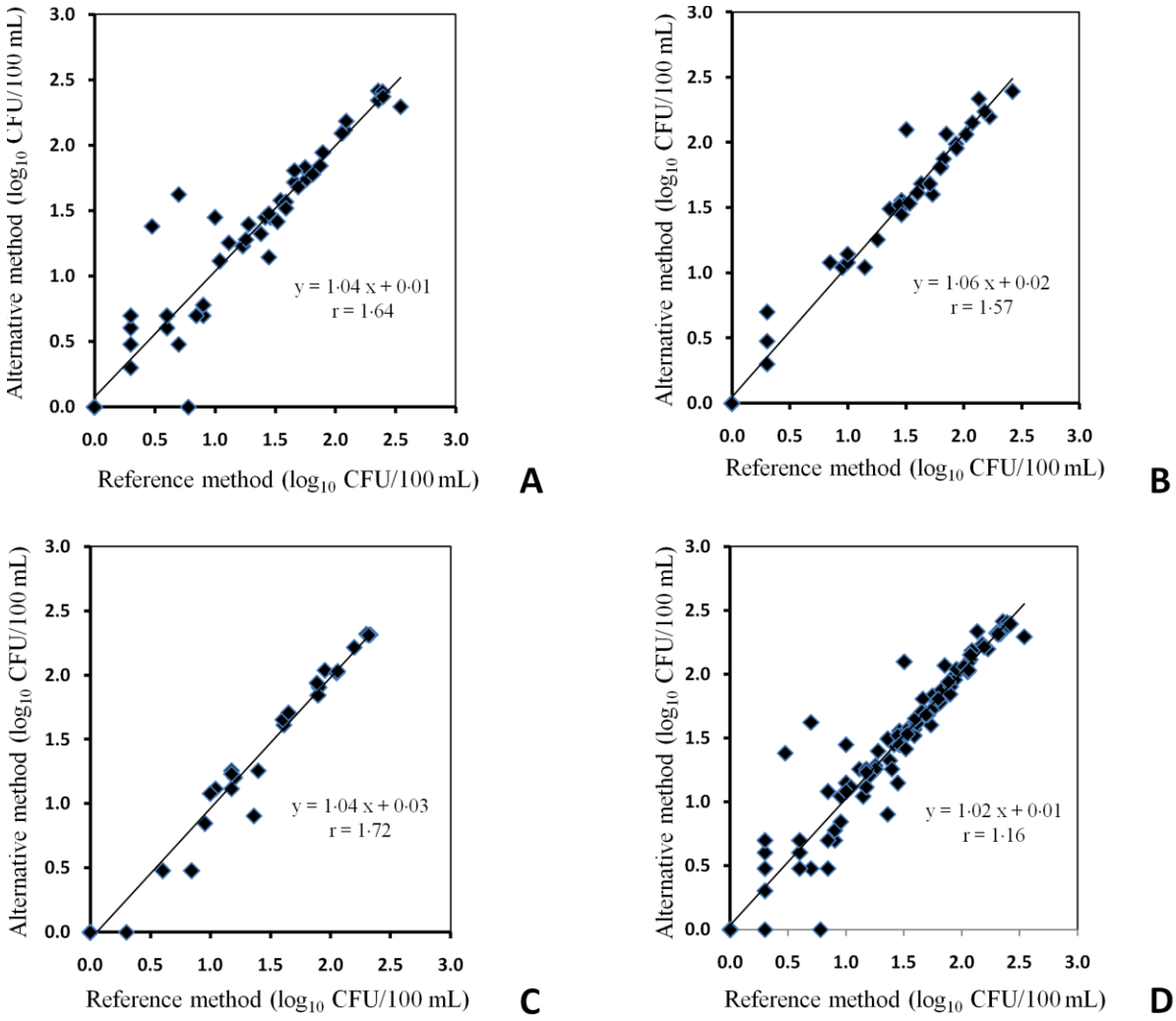

Figure 1. Scatter plot and orthogonal regression analysis results of $\log_{10}$ cfu per 100 ml of enterococci obtained by alternative versus reference methods for different water matrices: (A) Industrial water samples; (B) potable drinking water samples; (C) recreational water samples; (D) overall water samples. Regression is represented by the continuous line.

for the examination of several types of water samples, such as potable, industrial, and recreational waters, showing a good selectivity and a high efficiency.

The MF technique used in this study allowed the appropriate analysis of these water samples. This procedure has been the method of choice for the detection and enumeration of indicator bacteria in water for many years (Windle-Taylor and Burman, 1964), because it reduces the workload in laboratories when processing a large number of samples. In addition, several statistical studies have demonstrated that this technique is more accurate than the MPN method (APHA, 2012).

Given that enterococci are often associated with a diverse microbiota, selective media are needed for their isolation. Most of these media lack sufficient selectivity, which is necessary to distinguish clearly enterococci from the accompanying microbiota (Domig et al., 2003). In this case, the alternative method showed an appropriate selectivity in relation to the reference one. The value of this parameter was significantly higher than the acceptance value (-1) suggested by the ISO/TR 13843 standard (Messer and Dufour, 1998). The percentage of non-typical colonies, obtained on m-CromoCen ENT, indicates that the chromogenic medium was able to inhibit the accompanying microbiota and foster the growth of enterococci. The rates of false-positive and false-negative samples obtained are well below those reported for other chromogenic method for similar purposes (Messer and Dufour, 1998; Manafi, 2000). Some chromogenic media used for the isolation and preliminary identification of *Streptococcus* strains in clinical samples reported a high rate of false positives(Tibbs and Creighton, 2013). In addition, the efficiency achieved with the m-CromoCen ENT was slightly higher than that of the reference method (97%).

Table 3. Contingency table (2 × 2) built to verify the hypothesis that both methods have the same LOD.

Contingency table (2 x 2)			
	Reference	**Alternative**	**Population**
Positives	3	1	4
Negatives	3	5	8
Total	6	6	12

Recent studies with Enterolert, another rapid quantitative detection method of enterococci in the waters, reported a selectivity of -0.3 and 97% of accuracy, therefore it is less selective and less accurate than the m-CromoCen ENT (IDEXX, 2010).

Inclusivity is defined as the ability of a medium to detect the target microorganism from a wide range of strains, being related to with the sensitivity of the analytical method (ISO 16140, 2003). In this study, the inclusivity assay demonstrated a good sensitivity of the chromogenic method to detect enterococci from water samples. In addition, the results of the exclusivity test showed the lack of interference from a relevant range of non-target strains with the alternative method, demonstrating a good specificity of the method.

The most prevalent species isolated from all water sources were *E. faecalis* followed by *E. faecium*. Previous studies have reported that *E. faecalis* and *E. faecium* are the most frequently enterococci identified in water (Bennani et al*.,* 2012). The regression analysis for enterococci demonstrated a good correlation between both methods. The values of the intercept and the slope obtained for all water matrices studied verified a high degree of linearity.

A reason for higher counts obtained in m-CromoCen ENT was probably based on the ability of this medium to recover injured or stressed target microbial cells due to the absence of sodium azide and the inclusion of highly nutritive peptones and extracts in the formulation. The standard procedure medium includes this inhibitor and it may partially inhibit the growth of the target organisms, particularly if they are sub-lethally injured (Winter et al., 2012). Other chromogenic methods also have shown higher counts in comparison with conventional method (Wohlsen, 2011).

Another possible cause of this difference in recovery may be explained by the high incubation temperature recommended by the reference method that generally reduces the number of enterococci recovered; some strains do not grow or do not grow well at this temperature (Fisher and Phillips, 2009). Another inconvenience was observed when counting on colonies in the BAA agar: the esculin resulting complex spread throughout the medium when this compound was hydrolyzed by enterococci, thus creating difficulties in distinguishing target colonies within a mixed culture and interfering with the counting of enterococci colonies. Levin et al*.* (1975) reported that diffusion of the precipitate through this medium makes colony counting difficult, if not impossible, mainly when large numbers of colonies are present.

The graphs (Figure 1) illustrate that a greater dispersion of the counts was obtained at lower microbial concentration in all samples. Such findings can be explained by the distribution followed by microorganisms in a sample (Poisson distribution) (Niemelä, 1997). In random distribution, at lower levels, the probability of finding microorganisms in the sample is lower, in other words, the smaller the number of colonies in a sample the greater the uncertainty of measurements (ISO 13843, 2000).

In all cases, the regression lines suggest a strong correlation and an adequate relative accuracy between both methods. A previous work (Noble et al*.*, 2010), where the Enterolert method was compared with the EPA 1600 method for enterococci enumeration in recreational waters, reported a correlation coefficient of $r = 0.87$ and a slope of 0.92, values similar to those reported in the present study. However, a previously published paper about the Enterolert method for the detection of enterococci in recreational waters reported a lower correlation coefficient ($r = 0{\cdot}68$) (Eckner, 1998). Other rapid methods (PCR) that have been evaluated for the quantification of other bacteria in food reported correlation coefficient comparable to results reported in current experiments (Macé et al., 2013).

The detection limit attained was low but achieved detection of enterococci in most water samples. Another culture method, EPA 1600, claims a detection level of 2.3 cfu/100 ml, a value higher than that obtained in the present study (Maheux et al*.*, 2011). One more study, in which the chromogenic medium m-EI agar was used for the detection and enumeration of enterococci in seawater reported a LOD of 1 cfu/100 ml (Yamahara et al*.*, 2009). Another rapid enterococci detection method in water based on molecular biology has reported values above the detection limit of the present study; however, this technique requires several steps, increasing the overall cost (Maheux et al*.*, 2011). Chromogenic media for detection of other pathogens in spiked potable water samples showed a detection limit (1-20 cfu/100 ml) (Al-Wasify et al., 2013).

The new alternative method improves the LOD with regard to other existing methods for this purpose and allows enumeration of target bacteria even at low enterococci concentrations in the water samples.

The LOQ of the alternative method was also lower than that of the reference method. The capacity to recover enterococci using the m-CromoCen ENT is slightly higher than that of the reference method. Other media used for the enumeration of other bacteria in water samples have yielded a limit quantification of 1×10^2 cfu/l, a value higher than that obtained with the medium tested herein (Fitipaldi et al*.*, 2010).

The overall results of the comparative study of the new alternative chromogenic method showed that it is suitable for detecting and counting enterococci in water samples.

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REFERENCES

Al-Wasify RS, Al-Sayed AA, Kamel MM (2013). Sensitivity and specificity of chromogenic media for detection of some pathogens in water. Int. J. Environ. Sustainability. 2:1-9.

American Public Health Association (APHA) (2012). Standard methods for the examination of water and wastewater. 22nd ed, APHA, Washington DC, USA, pp. 88-90.

Asociación Española de Normalización y Certificación (2001). Calidad del agua. Detección y recuento de enterococos intestinales. Parte 2: Método de filtración por membrana, UNE-EN ISO 7899-2:2000. AENOR, Madrid, España.

Bennani M, Amarouch H, Oubrim N, Cohen N (2012). Identification and antimicrobial resistance of fecal Enterococci isolated in coastal mediterranean environments of Morocco. Eur. J. Sci. Res. 70:266-275.

Chang S, Lamm SH (2003). Human health effects of sodium azide exposure: a literature review and analysis. Int. J. Toxicol. 22:175-186.

Demircan A, Ozsarac M, Karamercan MA, Doğan NO (2011). Following accidental low dose sodium azide ingestion - Case Report. J. Academic Emergen. Med. 10:41-42.

Domig KJ, Mayer HK, Kneifel W (2003). Review article: Methods used for the isolation, enumeration, characterization and identification of *Enterococcus* spp. Media for isolation and enumeration. Int. J. Food Microbiol. 88:147-164.

Eckner KF (1998). Comparison of MF and MPN multiple-tube fermentation by the Colilert and Enterolert methods for detection of waterborne coliform bacteria, *Escherichia coli*, and enterococci used in drinking and bathing water quality monitoring in Southern Sweden. Appl. Environ. Microbiol. 64:3079-3083.

Fisher K, Phillips C (2009). The ecology, epidemiology and virulence of *Enterococcus*. Microbiology 155:1749-1757.

Fittipaldi M, Codony F, Morató J (2010). Comparison of conventional culture and real-time quantitative PCR using SYBR green for detection of *Legionella pneumophila* in water samples. Water SA. 36:417-424.

Frahm E, Obst U (2003). Application of the fluorogenic probe technique (TaqMan PCR) to the detection of *Enterococcus* spp. and *Escherichia coli* in water samples. J. Microbiol. Meth. 52:123-131.

Gander RM, Cavuoti D, Alatoom A, Southern P Jr, Grant D, Salinas K, Gaffney D, MacKenzie J, Byrd L (2013). Pseudo-outbreak of Vancomycin-Resistant-Enterococcus (VRE) colonization in a neonatal intensive care unit using spectra VRE surveillance medium. J. Clin. Microbiol. 51:810-813.

González MI, Torres T, Chiroles S, Manafi M (2009). Medios de cultivo fluorogénicos y cromogénicos y su evaluación en aguas de consumo y costeras. Hig. Sanid. Ambient. 9:422-430.

Hajia M, Rahbar M, Zade MM (2012). A novel method “CHROMagar” for screening vancomycin-resistant enterococci (VRE) isolates. Afr. J. Biotechnol. 11:9865-9868.

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (1994). Gram-positive cocci. In: Hensyl WR (eds) Bergey’s Manual of determinative bacteriology. 9th ed, Williams and Wilkins, Baltimore, USA. pp. 528-558.

IDEXX (2010). An ISO/TR 13843 Method performance validation for the Enterlolert*-DW and Quanti-Tray* System for the quantitative detection of enterococci in drinking water. Available at: http://www.idexx.com/pubwebresources/pdf/en_ca/water/8980-01-enterolert-dw-validation-report-final.pdf. [Accessed 12 March 2012].

ISO 13843 (2000). Water quality - Guidance on validation of microbiological methods, ISO/TR 13843:2000. International Organization for Standardization, Geneva, Switzerland.

ISO 16140 (2003). Microbiology of food and animal feeding stuffs-protocol for the validation of alternative methods, NF ISO 16140:2003. International Organization for Standardization, Geneva, Switzerland.

Levin MA, Fisher JR, Cabelli VJ (1975). Membrane filters technique for enumeration of enterococci in marine waters. Appl. Microbiol. 30:66-71.

Macé S, Mamlouk K, Chipchakova S, Prévost H, Joffraud JJ, Dalgaard P, Pilet MF, Dousset X (2013). Development of a rapid real-time PCR method as a tool to quantify viable *Photobacterium phosphoreum* bacteria in salmon (Salmo salar) steaks. Appl. Environ. Microbiol. 79:2612-2619.

Maheux AF, Bissonnette L, Boissinot M, Bernier JLT, Huppé V, Bérubé E, Boudreau DK, Picard FJ, Ann H, Michel GB (2011). Method for rapid and sensitive detection of *Enterococcus* sp. and *Enterococcus faecalis/faecium* cells in potable water samples. Water Res. 45:2342-2354.

Manafi M (2000). New developments in chromogenic and fluorogenic culture media. Int. J. Food Microbiol. 60:205-218.

Messer JW, Dufour AP (1998). A rapid, specific membrane filtration procedure for enumeration of enterococci in recreational water. Appl. Environ. Microbiol. 64:678-680.

Niemelä SI (1997). Performance characteristics of microbiological water analytical methods. European training courses on water quality measurements. Course A: Monitoring and measurements of lake recipients. Aug 25-29, Helsinki, Finland.

Noble RT, Blackwood D, Griffith JF, McGee CD, Weisberg SB (2010). Comparison of rapid quantitative PCR-based and conventional culture-based methods for enumeration of *Enterococcus* spp. and *Escherichia coli* in recreational waters. Appl. Environ. Microbiol. 76:7437-7443.

NordVal Validation (2009). Protocol for the validation of alternative microbiological methods. NV-DOC.D-2009-03-24. Available at: http://www.nmkl.org/NordVal/NordValprotocolmarch2009.pdf. [Accessed 12 March 2012].

Orenga S, James AL, Manafi M, Perry JD, Pincus DH (2009). Enzymatic substrates in microbiology. J. Microbiol. Meth. 79:139-155.

Pepe MT, Risso WM, dos Santos FA, Martone-Rocha S, Karmann V, da Silva TF, Alves MR (2011). Quality of water sources used as drinking water in a Brazilian Peri-urban area. Braz. J. Microbiol. 42:560-566.

Perry JD, Morris KA, James AL, Oliver M, Gould FK (2007). Evaluation of novel chromogenic substrates for the detection of bacterial *β*-glucosidase. J. Appl. Microbiol. 102:410-415.

Tibbs C, Creighton J (2013). A comparison of four comercial chromogenic media and blood agar for the isolation and preliminary identification of *Streptococcus agalactiae* from vaginal swabs. N. Z. J. Med. Lab. Sci. 67:52-55.

Volterra L, Bonadonna L, Aulicino FA (1985). Comparison of methods to detect fecal *streptococci* in marine waters. Water Air Soil Poll. 26:201-210.

Windle-Taylor E, Burman NP (1964). The application of membrane filtration techniques to the bacteriological examination of water. J. Appl. Bacteriol. 27:294-303.

Winter C, Kerros M-E, Weinbauer MG (2012). Effects of sodium azide on the abundance of prokaryotes and viruses in marine samples. PLoS One 7:e37597.

Wohlsen TD (2011). Comparative evaluation of chromogenic agar CM1046 and mFC agar for detection of *E. coli* and thermotolerant coliform bacteria from water samples. Lett. Appl. Microbiol. 53:155-160.

Yamahara KM, Walters SP, Boehm AB (2009). Growth of enterococci in unaltered, unseeded beach sands subjected to Tidal Wetting. Appl. Environ. Microbiol. 75:1517-1524.

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Full Length Research Paper

Effect of nodulating bacteria on the seed germination of *Capsicum* spp.

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The genus *Burkholderia* includes different species with biotechnological characteristics of great interest such as the degradation of xenobiotics, biological control, nitrogen fixation in free-living and symbiotic and phytohormone production. The diversity and versatility of this group of species is largely unexplored, including the production of secondary metabolites, which may have important practical applications. Therefore, the aim of this study was to evaluate the effect of seed germination of genotyes jalapeño and manzano peppers, inoculated with nodulating strains *Burkholderia sabiae* and 5B1. The outcome measures were percentage, index and coefficient of emergence. The results show that there is an increase in seed germination of aproximately 100% chili peppers jalapeño and 50% in manzano, inoculated with strain *B. sabiae*. Besides the increase in germination of seeds by *B. sabiae* strain, another feature of great biotechnological interest in this strain was also detected, which is the production of indoles. Based on these results, it is suggested that this strain can be used as an alternative for production of biofertilizers

Key words: Capsicum, germination, seeds, *Burkholderia*.

INTRODUCTION

The cultivation of *Capsicum* commonly known as pepper and one of the most important crops have increased due to demand in Mexico for the multiple uses of the fruits and it is naturally processed. It is also used as a condiment or as raw material for obtaining oleoresin dyes and industrial purposes as well as being a dietary source of antioxidants due to the content of flavonoids, phenolic compounds, carotenoids, ascorbic acid, vitamin A and capsaicinoids (Matsufuji et al., 1998; Osuna-García et al., 1998; Lee et al., 1995; Howard et al., 2000). The center of origin of *Capsicum* spp. is South America. The number of wild species comprising the genus *Capsicum* is between 20 and 30. Others authors mentioned about of 27 or 30 (Eshbaugh, 1982; Loaiza-Figueroa et al., 1983; Hernández et al., 1999; Moran et al., 2004) and they are only four or five domesticated species of *Capsicum* grown in the world. Mexico is the domestication center of five species: *Capsicum annuum* var. *annuum*, *Capsicum chinense*, *Capsicum pubescens*, *Capsicum* var *baccatum pendulum*, and the semi-domesticated *Capsicum frutescens*, and *Capsicum annu*um var. *glabriusculum* (Loaiza-Figueroa et al., 1983; Moran et al., 2004). In Mexico, there are wild populations of *C. annu*um and *C. frutescens*, which show great morphological and genetic variability (Hernández et al., 1998), and the species *Capsicum ciliatum* and *Capsicum lanceolatum*. *C. ciliatum* is found throughout the country except the Northwest, while *C. lanceolatum* has been reported only in the states of Chiapas and Veracruz (Hernandez-Verdugo et al., 1998).

The inoculation of seeds or seedlings with microbial inoculants has been adopted as a method for modifying microbial populations around crops plants to promote both development and yield. The stimulation of seedling

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development by bacteria has also been attributed to the production of biological active compounds. A significant increase in grain yield was also recorded in rice plants inoculated with plant growth promoting bacteria (Mantelin and Touraine, 2004; Mishra et al., 2006; Yang et al., 2009; López-Bucio et al., 2007). Similarly, inoculation of rhizobia to rice produced significantly higher roots and shoots biomass; increased their photosynthetic rate and accumulated higher levels of indoleacetic acid and gibberellin; phytohormones that regulator the growth. Plant growth promoting bacteria (PGPB) are bacteria that improve plant growth when introduced onto seeds, seed pieces, roots or into soil. The PGPB improve plant growth by one or more mechanisms: direct stimulation of plant growth by the production of phytohormones, vitamins and siderphores; enhancement of nutrient uptake; suppression of plant pathogens and/or induction of resistance in plant hosts against pathogens (Dakora and Phillips, 2003; Dobbelaere et al., 2003; Persello-Cartieaux et al., 2003; Mayak et al., 2004; Alikhani et al., 2006). *Burkholderia* species are characterized by their versatility, and by their ubiquity and diversity in both niches and environments. Most of the species belonging to the emerging beneficial-plant-environmental (PBE) *Burkholderia* group share important features, which provide them with advantages in their association with plants and with their immediate environments. The diversity and versatility of this group of species is largely unexplored, and this includes their production of secondary metabolites, which might have important practical applications (Suárez-Moreno et al., 2012).

Although β-rhizobia are particularly associated with the genus *Mimosa* and some related genera, they also nodulate several agriculturally important papilionoid legumes, including common bean (*Phaseolus vulgaris*) and honeybush tea (*Cyclopia* spp.), thus raising the possibility that they could be used as agricultural inoculants when their particular characteristics (e.g., tolerance to extreme pH, high salt tolerance) make them more suited to specific environments, such as in Morocco (Talbi et al., 2010) and the South African Cape (Elliott et al., 2007; Gyaneshwar et al., 2011). Therefore, the aim of this study was to evaluate the effect of seed germination of genotypes jalapeño and manzano peppers, inoculated with nodulating strains *Burkholderia sabiae* and 5B1.

MATERIALS AND METHODS

The experiment was conducted in the Biotechnology and Microbiology Laboratory, Universidad Tecnológica de la Selva, Chiapas, México. The cultivar *Capsicum* spp. used in this study was genotypes manzano and jalapeño. The seeds were collected from wild genotypes of *Capsicum* spp. in the regions of Palenque and Ocosingo, the State of Chiapas, México.

Plant growth promoting bacteria

Nodulating bacteria *Burkholderia sabiae* was provided by Ph.D Paulina Estrada de los Santos, from the Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, México. 5B1 strain was isolated from a legume native forest area of Chiapas. The design of the experiment was completely randomized with three replications (Table 1).

Table 1. Effect of inoculation of two strains nodulating bacteria, on the percentage of seed germination of chili, genotypes jalapeño and manzano after 14 days of inoculation.

Treatment	Germination after 14 days (%)	
	Jalapeño	Manzano
Control	35.0b	46.7b
Burkholderia sabiae	75.0a	68.5a
5B1	36.7b	50.0b

Means having different letters are significantly different at 5% level of significance.

Indol-acetic acid determination

Strains *B. sabiae* and 5B1 were evaluated based on production of indoleacetic acid, which were brown in NFB medium liquid for 18 h at 200 rpm, the inoculated cultures were adjusted to an optical density (OD) 0.2, and inoculated on media Jain and Patriquin culture with and without tryptophan. Once inoculated, they were incubated at 30°C for 24 and 48 h at 200 rpm. Subsequently, aliquots of 600 µl culture media were centrifuged at 5000g for 5 min. The presence of indol-3-acetic acid (IAA) in the supernatant was assayed according to the standard method modified from Rahman et al. (2010) in which the presence of the hormone in the culture reacts with Salkowski reagent.

Inocula preparation

Both bacterial strains were grown in peptone yeast extract (PY) and nutrient broth. Exponentially growing cells in shaken broth culture were inoculated. Chili pepper seeds were surface sterilized using etanol at 70% an Erlenmeyer flask and were treated with 7% sodium hypochlorite for 5 min followed by six times washing with sterile water. After that, the seeds were soaked in various nodulating bacteria. Seeds soaked in normal broth were treated as control. Twenty (20) seeds were inoculated, and the controls was put in sterilized Petri dishes containing agar 1% and kept at 24°C for 14 days.

Seedling emergence test

After soaking, the air-dried seeds were used for germination and the seedling percent emergence was calculated with the following formula:

$$\text{Emergence } \% = \frac{\text{Number of emerged seedlings}}{\text{Number of seeds sown}} \times 100$$

Germination speed

Copeland (1976) considers both vigor index and coefficient of

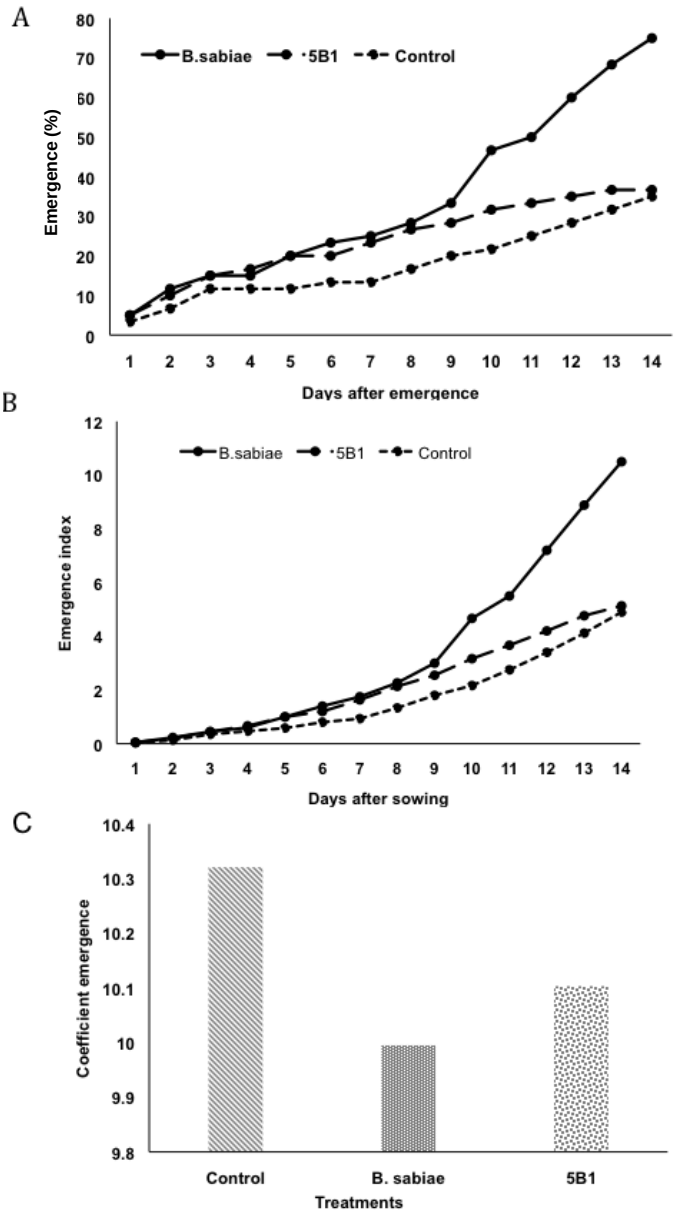

Figure 1. Effect of inoculation of *B. sabiae* and 5B1 bacteria on A, Percentage emergence; B, Emergence index; C, Coefficient emergence of seed germination of chili genotype jalapeño.

germination as measures for speed of germination. The germinated seedlings were counted at an interval of 24 h for 14 days and the speed of germination of seed was monitored. Coefficient of emergence and index emergence were calculated using the following formula (Copeland, 1976):

$$\text{Coefficient of emergence (\%)} = \frac{100(A_1+A_2+A_3+\text{----------}+A_X)}{A_1T_1+A_2T_2+\text{----------}+A_XT_X} \times 100$$

Where, A= number of seed germinated, T = time corresponding to A and x = numbers (1, 2, 3 ...nth).

$$\text{Emergence index} = \frac{\text{Ti Ni}}{\text{S}}$$

Where, Ti = ith number of days after sowing, Ni = ith number of seeds emergence, and S = total number of seed used.

Statistical analysis

Treatments were arranged in a randomized design. The analysis of variance and the LSD were calculated by using SAS package, Version 9.0 (SAS Institute Inc., 2006). Means were compared using the Tukey test at 5% level of significant.

RESULTS

Test germination

The effect of the germination of seeds of two genotypes of native peppers from the forest region of Chiapas, inoculated with strains of nodulating bacteria was evaluated (Figure 1A). Jalapeño genotype had the highest percentage of germination when seeds were inoculated with *B. sabiae* (75%), while strain 5B1 germination percentage was 36.7% (Figure 1A).

The statistical analysis for the comparison test of means by Tukey (5%) showed a significant difference in the treatment of *B. sabiae* with respect to control (Table 1). However, the results of the effect on germination of chili seeds genotype manzano, show that the germination percentage is 68.5% with *B. sabiae* strain (Figure 2A), whereas the percentage germination of seeds inoculated with 5B1 strain was 46.7% (Figure 2A), the statistical analysis for the comparison test of means by Tukey 5% showed a significant difference in *B. sabiae* treatment as compared to the control.

These results present a very similar behavior of *B. sabiae* and 5B1 in the germination of the two genotypes jalapeño and manzano (Table 1).

DISCUSSION

As shown in the results, there was an increase in the percentage of germination of the seeds of genotypes manzano and jalapeño, when inoculated with the strain of *B. sabiae*, the increase in the percentage of germination is a typical response of phytohormones gibberellins. Other parameters evaluated to determine the rate of germination of the seeds of *Capsicum* spp., was the effect of nodulating bacteria with germination coefficient (Figures 1B and 2B) and germination rate (Figures 1C and 2C).

As seen in the results, both parameters indicate that *B. sabiae* improves the speed of germination of chili seeds genotypes jalapeño and manzano. Although both strains are nodulating bacteria they both have an effect on the germination of seeds of *Capsicum* spp., which is a cha-

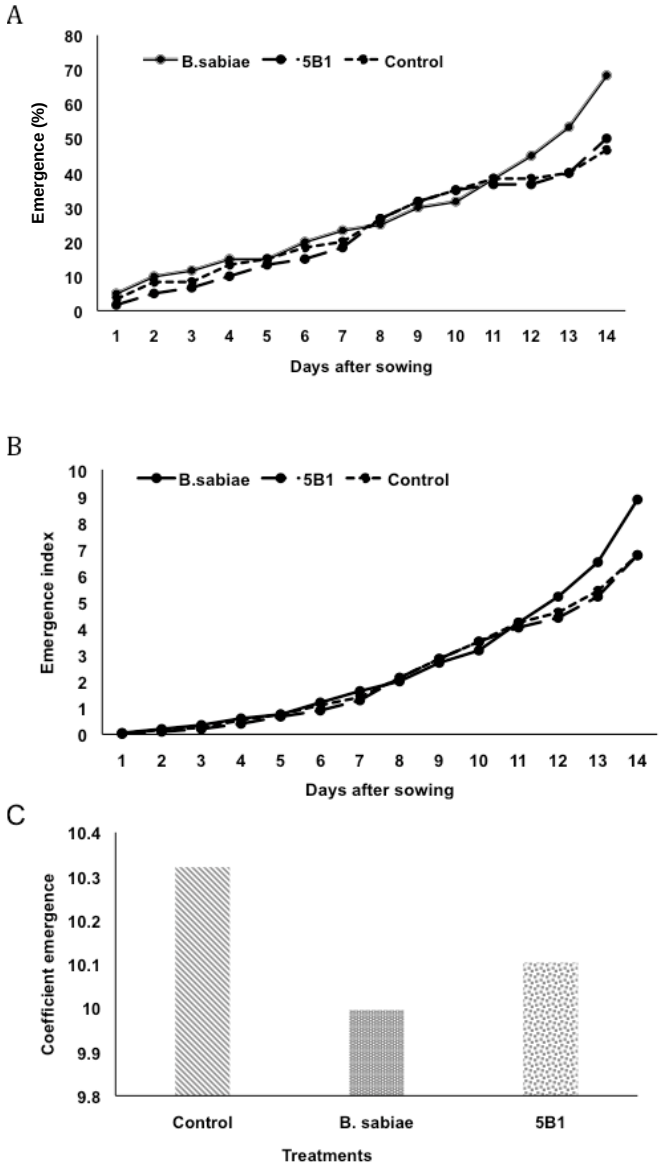

Figure 2. Effect of *B. sabiae* and 5B1 bacteria inoculation on seed. A, Percentage emergence; B, Emergence index; C, Coefficient emergence of germination of chili genotype manzano.

characteristic of great biotechnological interest, because they not only have a beneficial effect through the biological nitrogen fixation in symbiosis with nodulating legumes, but also stimulate germination in plants that do not form nodules as it is the case of *Capsicum* spp.

The effect on the seed germination could be due to a mechanism similar to that described in the rhizobia, they are released into the environment nod factors for leguminous plants, which stimulate the formation of nodules; but for non-legumes it has a stimulating effect on the germination of seeds. This affect rhizobia to stimulants the germination of seeds of leguminous plants that seems to be very similar in nodulating bacteria of *Burkholderia* genus (Antoun et al., 1998; Zhang and Smith, 2001; Dakora and Phillips, 2002; Smith et al., 2002). A fact of great importance is the production of indoles by *B. sabiae* (Data not show), as well as the ability of this strain to stimulate the germination of seeds of non-leguminous plant. The results of this study suggest that nodulating bacteria of *Burkholderia* genus increase the seed emergence and seedling vigor in seeds of *Capsicum* spp.

Also, it constitutes an economic methodology that can be used to optimize the germination rate, uniformity, and final percentages, features that directly affect crop production (Di Barbaro et al., 2005). With these characteristics and the results obtained, we proposed that this strain can be used as an alternative for biotechnological production of biofertilizers.

REFERENCES

Antoun H, Beauchamp CJ, Goussard N, Chabot R, Lalande R (1998). Potential of Rhizobium and Bradyrhizobium species as plant growth promoting rhizobacteria on non-legumes: Effects on radishes (*Raphanus sativus* L.). Plant Soil 204:57-67.

Copeland LO (1976). Principals of Seed Science and Technology. Burgess publishing company, Minnaeapolis, Minnesota. pp. 164-165.

Dakora FD, Phillips DA (2002). Root exudates as mediators of mineral acquisition in low-nutrient environments. Plant Soil 245:35- 47.

Di Barbaro G, Pernasetti S, Stegmayer A (2005). Effects evaluation of *Azospirillum brasilensis* inoculation on pepper (*Capsicum annum* L.var. trompa de elefante) seeds germination and plants emergence. Revista del CIZAS. 74:74-85

Dobbelaere S, Vanderleyden J, Okon Y (2003).Plantgrowth-promoting effects of diazotrophs in the rhizosphere. Crit. Rev. Plant Sci. 22:107-149.

Elliott GN, Chen WM, Bontemps C, Chou JH, Young JPW, Sprent JI, James EK (2007). Nodulation of Cyclopia spp. (Leguminosae, Papilionoideae) by *Burkholderia tuberum*. Ann. Bot. 100:1403-1411.

Eshbaugh WH (1982). Variation and evolution in *Capsicum eximium* Hunz. Baileya 21:193-198.

Gyaneshwar P, Hirsch AM, Moulin I, Chen WM, Elliott GN, Bontemps C, Estrada de Los Santos P, Gross E, Dos Reis Junior FB, Sprent JI, Young JP, James EK (2011). Legume-nodulating betaproteobacteria: diversity, host range and future prospects. Mol Plant Microbe Interact. 11:1276-1288.

Hernández VS, Dávila A, Oyama K (1999). Síntesis del Conocimiento Taxonómico, Origen y Domesticación del Género Capsicum. Boletín de la Sociedad Botánica de México 64:65-84.

Hernández-Verdugo S, Guevara-González RG, Rivera-Bustamante RF,. Vázquez-Yanes C, Oyama K (1998). Los parientes silvestres del chile (*Capsicum* spp.) como recursos genéticos. Boletín de la Sociedad Botánica de México 62:171-181.

Howard LR, Talcott ST, Brenes CH, Villalon B (2000). Changes in Phytochemical and Antioxidant Activity of Selected Pepper Cultivars (*Capsicum* Species) As Influenced by Maturity. J. Agric. Food Chem. 48(5):1713-1720.

Lee Y, Howard LR, Villalon B (1995). Flavonoids and antioxidant activity of fresh pepper (*Capsicum* annuum) cultivars. J. Food Sci. (USA). 60(3):473- 476.

Loaiza-Figueroa F, Ritland K, Laborde-Cancino JA, Tanksley SD (1989). Patterns of genetic variation of the genus Capsicum (Solanaceae) in Mexico. Plant Syst. Evol. 165: 159-188.

López-Bucio J, Campos-Cuevas JC, Hernández-Calderón E, Velásquez-Becerra C, Farías-Rodríguez R, Macías-Rodríguez LI, Valencia-Cantero E (2007). *Bacillus megaterium* rhizobacteria promote growth and alter root system architecture through an auxin and ethylene independent signaling mechanism in *Arabidopis thaliana*. Mol. Plant-Microbe Interact 20:207-217.

Mantelin S, Touraine B (2004). Plant growth-promoting bacteria and

nitrate availability: impacts on root development and nitrate uptake. J Exp. Bot. 55:27-34.
Matsufuji H, Nakamura H, Chino M, Takeda M (1998). Antioxidant activity of capsanthin and the fatty acid esters in paprika (*Capsicum annuum*). J. Agric. Food Chem. 46:3468-3472.
Mayak S, Tirosh T, Glick BR (2004). Plant growth promoting bacteria that confer resistance in tomato to salt stress. Plant Physiol. Biochem. Plant Physiol. Biochem. 42(6):565-572.
Mishra RP, Singh RK, Jaiswal HK, Kumar V, Maurya S (2006). Rhizobium- mediated induction of phenolics and plant growth promotion in rice (*Oryza sativa* L.). Curr. Microbiol. 52:383-389
Morán BS, Ribero M, García F, Ramírez P (2004). Patrones isoenzimáticos de chiles criollos (*Capsicum annuum* L.) de Yucatán, México. En: Chávez-Servia, J.L., Tuxill, J., Jarvis, D.I. (eds). Instituto Internacional de Recursos Fitogenéticos. Cali, Colombia. pp. 83-89.
Osuna-García JA, Wall MM (1989). Prestorage moisture content affects color loss of ground paprika (*Capsicum annuum* L.) under storage. J. Food Qual. 21:251 259.
Persello-Cartieaux F, Nussaume L, Robaglia C (2003) Tales from the underground: Molecular plant-rhizobacteria interactions. Plant Cell Environ. 26:189-99.
Rahman A, Sitepu IR, Tang S, Hashidoko Y (2010). Salkowski´s reagent test as a primary screening index for functionalities of rhizobacteria isolated from wild dipterocarp saplings growing naturally on medium-strongly acidic tropical peat soil. Biosc. Biotechnol. Biochem. 74(11):2202-2208.
SAS Institute Inc. (2004). SAS/STAT Guide to personal Computers. SAS Institute Inc. Cary NC.
Smith DL, Prithiviraj B, Zhang F (2002). Rhizobial signals and control of plant growth. In: Finan TM, O'Brian MR, Layzell DB, Vessey K, Newton WE, eds. Nitrogen fixation: global perspectives. Wallingford, UK: CABI Publishing. pp. 327-330.
Suárez-Moreno ZR, Caballero-Mellado J, Coutinho BG, Mendonça-Previato L, James EK Venturi Vi (2012). Common Features of Environmental and Potentially Beneficial Plant-Associated Burkholderia. Microb. Ecol. 63:249-266.
Talbi C, Delgado MJ, Girard L, Ramirez-Trujillo A, Caballero- Mellado J, Bedmar EJ (2010). *Burkholderia phymatum* strains capable of nodulating *Phaseolus vulgaris* are present in Moroccan soils. Appl. Environ. Microbiol. 76:4587-4591.
Yang J, Kloepper JW, Ryu CM (2009). Rhizosphere bacteria help plants tolerate abiotic stress. Trends Plant Sci. 14:1-4.
Zhang F, Smith DL (2001). Interorganismal signalling in suboptimum environments: the legume-rhizobia symbiosis. Adv. Agron. 76: 125 - 161.

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Review

Effect of stress on intestinal microbiota of Arctic charr, Atlantic salmon, rainbow trout and Atlantic cod: A review

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The gastrointestinal (GI) tract of fish reacts to stress, sometimes with severe cell damage to intestinal enterocytes and modulation of the gut bacterial community. The effect of dominance hierarchy formation, acute - and handling stress on the intestinal bacterial community have been reported in three salmonids; Arctic charr (*Salvelinus alpinus* L.), Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss* Walbaum), and Atlantic cod (*Gadus morhua* L.). In Arctic charr, hierarchy formation reduced cultivable autochthonous (adherent) bacteria level in proximal intestine of subordinate fish compared to dominant fish, but this effect was not noticed in the distal intestine (DI). Furthermore, hierarchy formation modulates the gut microbiota. When Atlantic salmon and rainbow trout were exposed to acute stress the population level of adherent bacteria decrease in DI with concomitant increase in faeces, but this effect was not observed in Atlantic cod. The reason for the effect in Atlantic salmon is probably a peel-off effect of mucus and transporting autochthonous gut bacteria out of the fish. This will in turn allow allochthonous bacteria (present in the lumen or associated to digesta) to adhere and colonise the mucus layer. The elimination of the existing beneficial adherent gut microbiota and the lack of protecting mucus during acute stress might have relevance in pathogenesis. The present study reveals that stress eliminates certain protecting bacteria in the GI tract making the gut more prone to pathogen infections.

Key words: Fish, stress, intestinal microbiota.

INTRODUCTION

Stress in teleost fish is generally characterised by a set of changes in the normal metabolism. The stress response; thought to be compensatory and/or adaptive and enabling the animal to cope with stressors. Examples of stressors are; environmental changes (temperature and salinity), hauling, sorting, handling, transportation, pH stress and oxidative stress (Sandodden et al., 2001; Van Ham et al., 2003; Hoskonen and Pirhonen, 2006; Rollo et al., 2006; Torrecillas et al., 2012). Fish exposed to stress is known to have a major impact on; blood plasma (Biron and Benfey, 1994; Olsen et al., 2002; 2005; 2008), intestinal and enterocyte function; immediate damage to the junctional complexes evidenced by dissociation of both desmosomes and nexus (Peters, 1982; Szakolczai, 1997;

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Olsen et al., 2002; 2005), and increase the susceptibility to infectious diseases in fish (Wedemeyer, 1970; Snieszko, 1974; Mazeaud et al., 1977; Peters et al., 1988; Pickering and Pottinger, 1989).

In animals and humans, stress reduce the number of lactobacilli, beneficial bacteria, while there is an increase in epithelial adherence, prevalence and mucosal uptake of Gram-negative pathogens, for example, *Escherichia coli* and *Pseudomonas* (Tannock and Savage, 1974; Lutgendorff et al., 2008; Qiao et al., 2013), but the effect of different stress on the total bacteria level (CFU g^{-1}) and bacterial community in fish GI tract is less studied (Ringø et al., 1997; Ringø et al., 2000; Olsen et al., 2002, 2005, 2008). These studies revealed that fish exposed to stress affected both the total intestinal cultivable bacterial level and modulated the autochthonous (adherent) gut microbiota, those directly associated with the mucosa and mucus. Elucidation of the effect of stress on gut microbiota is of importance to evaluate as the elimination of the existing microbiota and loss of protecting mucus in stressed fish is likely to be important in pathogenesis of certain diseases that may use the GI tract as infection route (Groff and LaPatra, 2000; Birkbeck and Ringø, 2005; Ringø et al., 2010). If some of the existing bacteria present in the lumen are pathogenic, it is conceivable that they can adhere to membrane surfaces, result in mucosal tolerance or inflammation and translocate to infect otherwise sterile tissues and establish disease.

In addition to evaluation of studies published on the effect of stress on fish gut bacterial community, the present paper presents some data from unpublished studies having relevance with regard to the topic discussed.

EFFECT OF DOMINANCE HIERARCHY FORMATION ON GUT MICROBIOTA OF ARCTIC CHARR

Dominance hierarchy formation is a common feature of many fish species, and various aspects have been the subject of several studies (Sneddon et al., 2005; Paull et al., 2010) and is synonymous with access to food resources, shelter and/or reproduction. Within a hierarchy dominant individuals are more aggressive and grow markedly faster than subordinates, low-ranking individuals. To our knowledge, only one paper has been published on the effect of dominance hierarchy formation on fish gut microbiota (Ringø et al., 1997). In this study with Arctic charr, 373 autochthonous bacteria (aerobic and facultative aerobic) were isolated and enumerated from proximal intestine (PI) and distal intestine (DI) as described by Ringø (1993) and classified to genus level by standard biochemical tests as described by Muroga et al. (1987; Gram-negative bacteria) and Ringø (1993; Gram-positive bacteria). The study of Ringø et al. (1997) revealed that cultivable adherent bacterial level (CFU g^{-1}) in subordinate fish was reduced in the PI (log 3.48) compared to dominant fish; log 4.36. In contrast, no effect was noticed in the DI. Moreover, the composition of adherent bacteria was modulated as; *Aeromonas salmonicida* and *Xanthomonas* spp. were only detected in the GI tract of dominant fish, while bacteria belonging to the Cytophaga/ *Flexibacter* group were only detected in the digestive tract of subordinate fish. Furthermore, *Aeromonas hydrophila*, *Enterobacter* spp. *Vibrio fluvialis* and *Carnobacterium divergens* were only detected in DI of the subordinate fish and indicate that these bacteria genera are able to adhere and colonise the DI even when low feed intake occurs. Whether the modulation in the gut microbiota reported in this study affects the survival in challenge studies has not been elucidated and merits investigations.

EFFECT OF ACUTE STRESS ON GUT MICROBIOTA IN SALMONIDS

The intestinal mucus layer in fish has several important factors in fish health and welfare (Shepard, 1994). One important factor is the competitive exclusion of pathogens adhesion by intestinal bacteria (Ringø et al., 2005; Merrifield et al., 2014). Removal or disruption of the mucus layer therefore has the potential to open for pathogen adherence and loss of protective effect of the normal microbiota. In fish, the microbial communities have been shown to be essential for proper GI development and are implicit in aiding digestive function, immunological function and disease resistance (Gomez and Balcázar, 2008; Nayak, 2010).

In three studies with Atlantic salmon and rainbow trout evaluated the effect of stress on gut microbiota. Sampling of gut bacteria from control fish were carried out on fish not subjected to stress. The stress experiment was initiated by exposing the fish to acute stress by reducing the water level to 5-10 cm depth and subsequently chasing the fish with a pole for 15 min (Olsen et al., 2002; 2005; Zhou, He, Olsen and Ringø, unpublished data) and the gut microbiota were sampled according Ringø (1993); four hours post stress. The main effect of these stress studies was a peeling-off effect of mucus. In the study of Olsen et al. (2002) intestinal samples from mid intestine (MI), DI and faeces were collected for measurements of CFU g^{-1}. In fish subjected to stress, the population level of cultivable adherent gut bacteria (aerobic and facultative aerobic) in MI and DI were significantly reduced; from approximately log 4.2 prior to stress to ca. log 2.5 post stress, followed by a subsequent and significant increase in fecal CFU g^{-1}; from log 4.0 prior to stress to log 5.5 after stress. To confirm the results from cultivable bacterial analysis; electron microscopy evaluations of MI by methods described by Ringø et al. (2001) were carried out. Scanning electron microscopy evaluation; 4 h post stress revealed few bacteria at the enterocytes surface (Figure 1) compared to transmission

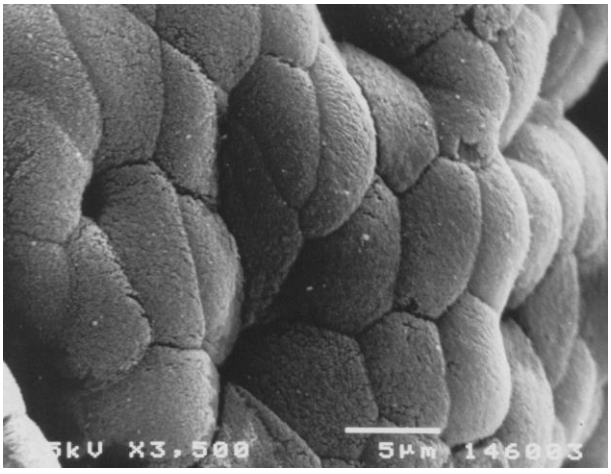

Figure 1. Scanning electron microscopy micrograph of the brush border membrane of midgut of Atlantic salmon 4 h after acute stress. Cell borders are clearly seen, but few bacteria are seen associated with the enterocytes surfaces. After Olsen, Myklebust and Ringø (unpublished data).

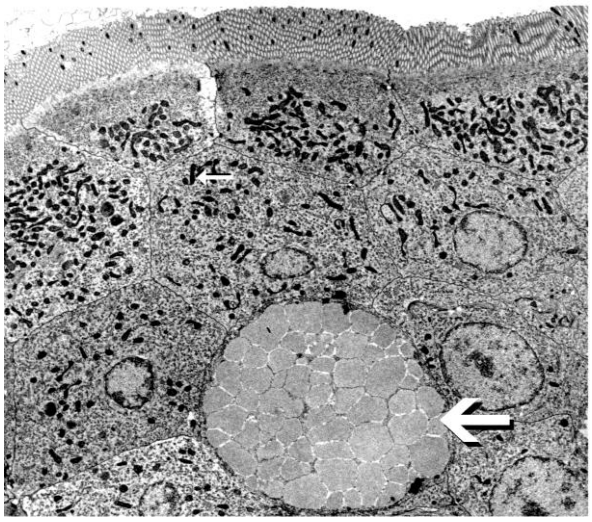

Figure 2. Transmission electron microscopy micrograph showing bacteria associated with the microvilli of enterocytes in the midgut of Atlantic salmon; not stressed fish. Small arrow - mitochondria; large arrow - goblet cell. After Ringø, Olsen and Myklebust and (unpublished data).

electron micrograph of a control fish where numerous bacteria are seen associated with the microvilli of enterocytes (Figure 2).

In the study of Olsen et al. (2005) totally 313 aerobic and facultative aerobic bacteria were isolated from unstressed and acute stressed rainbow trout. The population level of cultivable adherent aerobic and facultative aerobic bacteria (CFU g^{-1}) in DI decreased from log 4.3 (CFU g^{-1}) prior to stress to log 2.7 post stress, but increased in faeces from log 4.7 before stress to log 5.6 four hours post stress (Olsen et al., 2005). The concomitant bacterial increase in faeces post-stress revealed by Olsen et al. (2002, 2005) might have some relevance in the protection against other bacteria to adhere and colonise the intestinal mucosa. Furthermore, Olsen et al. (2005) reported elimination of some adherent bacteria genera in DI; *Acinetobacter* spp. and *Rhodococcus* spp., and a reduced level of *Pseudomonas* spp.; from log 3.79 to 2.62 CFU g^{-1}, and *Staphylococcus* spp.; from log 4.04 to log 1.10 CFU g^{-1}, prior to and post-stress, respectively. The increase in level of *Arthrobacter* and *Micrococcus* genera in faeces 4 h post-stress and their presence below detection level in the DI prior to stress might indicate that these bacteria genera either colonise the MI or they are weakness attached to the digestive tract mucosa and are lost during preparation; the intestines were rinsed three times in sterile 0.9% saline to remove non-adherent (allochthonous) bacteria.

A study devoted to evaluate adherent lactic acid bacteria (LAB) in the GI tract (PI, MI and DI) of fed Atlantic salmon exposed to regular (repeated) handling stress; reducing the water level to 5-10 cm depth and subsequently chasing the fish in the tank with a pole for 5 min (Ringø et al., 2000) revealed no significant reduction in LAB counts and LAB identified by phenotypic identification [Gram-staining, morphology, motility, production of catalase and oxidase, Huge and Leifson´s fermentation test, gas from glucose and growth on acetate agar (pH 5.4) and cresol red thallium acetate sucrose agar (pH 9.1)] and DNA sequence analysis in PI, MI or DI. In total 44, 36 and 35 strains were isolated from PI, MI and DI, respectively. Of these belonged 14, 8 and 8 isolates to LAB isolated from PI, MI and DI, respectively. As no further identification of gut bacteria not belonging to LAB was carried out; a further study evaluate the bacterial community in PI, MI and DI; sampled prior to stress and after 11 days of regular exposed to regular handling stress (Ringø, unpublished data). Removal of the GI tract from the fish is described in detail by Ringø (1993). Homogenates of the different gut segments were diluted in sterile saline solution and 100 µl of appropriate dilutions were spread onto the surface of two different tryptic soy agar plates as described by Ringø et al. (2000). Hundred and ninety-nine isolates were classified into 13 taxonomic groups by phenotypic identification as described by Ringø and Olsen (1999). A complex bacterial community was observed and included the Gram-negative genera; *Acinetobacter*, *Aeromonas*, *Alcaligenes*, Cytophaga/ *Flexibacter* - group, *Moraxella*, *Photobacterium*, *Pseudomonas*, *Xanthomonas* and the Gram-positive genera; *Brevibacterium*, *Microbacterium*, *Micrococcus*, LAB and *Staphylococcus*. These taxonomic groups were detected in PI, MI and DI prior to handling stress (Table 1). In contrast, after 11 days of regular handling stress only adherent *Acinetobacter*, *Pseudomonas*, *Xanthomonas* and LAB were isolated from the GI tract.

Table 1. Changes in log total viable counts (log TVC) of adherent cultivable bacteria genera present in proximal intestine (PI), mid intestine (MI) and distal intestine (DI) of Atlantic salmon (*Salmo salar* L.) prior to stress and after 11 days of regular stress. The log TVC values are means of six individual fish. After Ringø (unpublished data).

Bacteria	Prior to stress			11 days of regular stress		
	PI	MI	DI	PI	MI	DI
Log TVC	2.62	3.00	2.45	3.03	3.09	2.68
No. of isolates	44	36	35	25	28	31
Genus						
Acinetobacter spp.	1.76	1.74	1.51	2.63	2.60	2.14
Aeromonas spp.	1.76	n.d	n.d	n.d	n.d	n.d
Alcaligenes spp.	1.46	1.75	n.d	n.d	n.d	n.d
Cytophaga/*Flexibacter*-group	1.28	2.04	1.38	n.d	n.d	n.d
Moraxella spp.	n.d	n.d	0.90	n.d	n.d	n.d
Photobacterium spp.	n.d	1.44	n.d	n.d	n.d	n.d
Pseudomonas spp.	1.76	1.92	1.68	2.59	2.42	2.09
Xanthomonas spp.	n.d	1.74	1.51	n.d	2.49	1.89
Brevibacterium spp.	n.d	1.44	n.d	n.d	n.d	n.d
Microbacterium spp.	n.d	1.44	n.d	n.d	n.d	n.d
Micrococcus spp.	n.d	1.74	n.d	n.d	n.d	n.d
Staphylococcus spp.	1.76	2.40	1.68	n.d	n.d	n.d
LAB	2.13	2.35	1.81	2.24	2.24	2.03
Unknown*	0.98	1.44	1.38	1.64	1.94	1.49

11 days of regular stress had no significant effect on total viable counts of adherent bacteria in PI, MI and DI. n.d - not detected in the GI segment, LAB - lactic acid bacteria, * - died prior to positive identification.

This clearly showed that 11 days of regular handling stress modulates the autochthonous gut microbiota of Atlantic salmon. Based on these results we put forward the controversial hypothesis that the taxonomic groups; *Aeromonas*, *Alcaligenes*, Cytophaga/ *Flexibacter*, *Moraxella*, *Photobacterium*, *Brevibacterium*, *Microbacterium*, *Micrococcus*, and *Staphylococcus* are less strongly associated to the intestine and are lost during regular handling stress. This might open for opportunistic - and pathogenic bacteria to adhere and colonise the GI tract and initiate disease.

In the two salmonid studies of Olsen et al. (2002; 2005), the effect of stress on the intestinal microbiota was evaluated by conventional culture based methods. As conventional methods are time consuming and lack accuracy (Asfie et al., 2003) and sensitivity in characterizing certain fastidious and obligate anaerobes require special culture conditions, Zhou et al. (unpublished data) carried out an experiment where polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) analysis was used to evaluate the bacterial community in acute stressed Atlantic salmon fed fish meal and fish oil. The autochthonous and allochthonous bacterial community in DI of three individual fish was evaluated by PCR-DGGE analysis according to Liu et al. (2008). The band position and its intensity were analysis by Glyko Bandscan 5.0 (Glyko, Novato, CA, USA), and the relative abundance (peak area) within the sample profile were calculated to the total bands gray-scale value. Cluster analysis was performed using the Jaccard's coefficient of similarity and the un-weighted pair group method (UPGMA) using the NTSYS software package. Obtained sequences were searched for in the GenBank library BLAST to find the closest relative for the partial 16S rRNA gene. Nucleotide sequences were deposit in GenBank under the accession Nos. EU697150 - EU697178. The results revealed higher values of pairwise similarity coefficient (Cs) in allochthonous bacterial community between the non-stressed and stress fish than the autochthonous bacterial community (Table 2), which indicate that autochthonous bacteria were more likely affected by acute stress than allochthonous bacteria. Representatives of the autochthonous and allochthonous bacterial communities of three not-stressed and three stressed fish are presented in Table 3, respectively. Stress stimulated abundance of some potential pathogenic bacteria, such as allochthonous *Lactobacillus letivazi* (AJ417738; band 19); not detected in non-stressed fish, autochthonous *Vibrio* sp. B5-8a (DQ357798; band 13), *Vibrio logei* strain T2110 (DQ318955; band 14 and 15), *Vibrio* sp. BC-R3 (DQ357806; band 16), and uncultured *Pseudomonas* sp. (DQ189764; band 28); not detected in non-stressed fish. Furthermore, a generally higher abundance of

Table 2. Pairwise similarity coefficient (Cs) matrix for the intestinal bacterial community of three individual Atlantic salmon fed fish meal and fish oil; not-stressed and acute stressed fish. DI - distal intestine. After Zhou et al. (unpublished data)

	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12
L1	1.0											
L2	0.8	1.0										
L3	0.8	0.67	1.0									
L4	0.87	0.93	0.67	1.0								
L5	0.87	0.73	0.93	0.73	1.0							
L6	0.8	0.73	0.83	0.73	0.83	1.0						
L7	0.87	0.73	0.93	0.73	1.0	0.83	1.0					
L8	0.8	0.73	0.8	0.8	0.73	0.73	0.73	1.0				
L9	0.76	0.7	0.63	0.7	0.7	0.7	0.7	0.57	1.0			
L10	0.83	0.77	0.7	0.83	0.77	0.7	0.77	0.9	0.6	1.0		
L11	0.83	0.77	0.7	0.83	0.77	0.7	0.77	0.9	0.6	1.0	1.0	
L12	0.83	0.77	0.7	0.83	0.77	0.7	0.77	0.9	0.6	1.0	1.0	1.0

Unstressed fish (control); L1, autochthonous microbiota from DI of fish no. 1; L2, allochthonous microbiota from DI of fish no. 1; L3, autochthonous microbiota from DI of fish no. 2; L4, allochthonous microbiota from DI of fish no. 2; L5, autochthonous microbiota from DI of fish no. 3; L6, allochthonous microbiota from DI of fish no. 3. Stressed fish: L7, autochthonous microbiota from DI of fish no. 1; L8, allochthonous microbiota from DI of fish no. 1; L9, autochthonous microbiota from DI of fish no. 2; L10, allochthonous microbiota from DI intestine of fish no. 2; L11, autochthonous microbiota from DI of fish no. 3; L12, allochthonous microbiota from DI of fish no. 3

Flavobacterium sp. SOC A4 (DQ628956; band 29) was observed in stressed fish. Whether the modulation of gut bacterial community has any effect on fish health and disease resistance is not known and merits further investigations.

IN VITRO GROWTH INHIBITION OF TWO FISH PATHOGENS BY CARNOBACTERIA ISOLATED FROM THE GI TRACT OF ATLANTIC SALMON PRIOR TO AND POST STRESS

Antibacterial activity by bacteria isolated from aquatic animals and the environment has been revealed in numerous studies (e.g. Gatesoupe, 1999; Gram et al., 1999; Sugita et al. 2002) as well as information about LAB isolated from the GI tract fish (Jöborn et al., 1999; Ringø et al., 2005; Ringø, 2008). The 199 *Carnobacterium* strains isolated and identified by phenotypic identification and DNA sequence analysis from the GI tract of Atlantic salmon; before stress, 5 h after stress, 24 h and 11 days following of regular handling stress by Ringø et al. (2000), where further tested for their ability to inhibit *in vitro* growth of two fish pathogens; *Aeromonas salmonicida* subsp. *salmonicida* LFI 4038 (furunculosis) and *Moritella viscosa* LFI 5000 (winter ulcer) (Ringø, unpublished data). *In vitro* growth inhibition was carried out using the microtitre method described elsewhere (Ringø et al., 2005; Salma et al., 2011; Askarian et al., 2012). The test revealed that 139 carnobacteria isolates inhibited *in vitro* growth of *A. salmonicida* while only 21 isolates inhibited growth of *M. viscosa* (Table 4). 97.4% of the bacterial strains isolated from fish prior to handling stress showed growth inhibition towards *A. salmonicida*, while only 76.7 and 46.9% of the isolated bacterial strains from fish; 5 and 24 h after stress, respectively displayed this ability. There were no significant differences between the different gut regions with regard to carnobacteria's antibacterial activity towards *A. salmonicida*. After 11 days of daily handling stress; only 43.2% of the carnobacteria isolated had the ability to inhibit *in vitro* growth of *A. salmonicida*. A similar trend as noticed in the growth inhibitions tests towards *A. salmonicida* was seen in the ability of carnobacteria to inhibit *in vitro* growth of *M. viscosa*. Prior to stress, approximately 16% of the isolates inhibited growth of *M. viscosa*, but after 11 days of regular stress only one out of 37 carnobacteria was able to inhibit growth of the pathogen (Table 4). The reason for the difference in the antibacterial activities of carnobacteria towards *A. salmonicida* and *M. viscosa* might be due to different infection routes. Previous studies have reported that *A. salmonicida* infect Atlantic salmon and rainbow trout via the intestine (Ringø et al., 2004; Jutfelt et al., 2006), while the skin is the possible infection route of *M. viscosa* as the bacterium was isolated from a natural outbreak of winter ulcer.

The reason why the frequency of carnobacteria able to inhibit the two pathogens decreased during the experiment is not known and call for further studies. One hypothesis might be that some carnobacteria revealing antibacterial activity are less strongly associated to the epithelial mucosa. The loss of the beneficial carnobacteria strains colonising the intestine of Atlantic salmon may lead to adherence and colonisation of pathogens if they are present in the digestive tract when the stress is repeated over several days.

Table 3. Representative autochthonous and allochthonous bacterial community and their abundance in the DI of three individual Atlantic salmon not exposed to stress. Auto - autochthonous; Allo - allochthonous. After Zhou et al. (unpublished data)

Not-stressed fish	Closest relative (Blast search)	Identity (%)	Accession no.	Fish no. 1		Fish no. 2		Fish no. 3	
				Auto	Allo	Auto	Allo	Auto	Allo
Band no.	**Bacteroides**								
3	*Flavobacterium psychrolimnae*	99	AJ585428	4.3		4.4		3.8	4.8
8	*Flavobacterium* sp. SOC A4(51)	98	DQ628945	7.3	5.8	16.8	7.2	14.9	6.4
29	*Flavobacterium* sp. SOC A4(51)	100	DQ628945	2.8	3.5	5.6	4.7	5.1	6.3
	Firmicutes								
5	*Lactobacillus aviarius* subsp. *aviarius*	98	AB326355	3.1	13.5		10.9		6.7
17	*Lactobacillus aviarius*	98	AB175728		11.4		17.5		8.7
18	*Lactobacillus aviarius* subsp. *aviarius*	100	AB326355	11.0	9.3		9.6		
11	*Anoxybacillus* sp.	96	DQ452025			3.8			14.5
6	Uncultured *Lachnospiraceae* bacterium	99	EF705084		7.2	1.6		10.3	
	Proteobacteria								
7	*Vibrio* sp. B5-8a	100	DQ357798	5.3	18.9		16.5		13.1
13	*Vibrio* sp. B5-8a	100	DQ357798		2.7				
10	*Vibrio logei* strain T21110	97	DQ318955			1.6			4.9
1	*Pseudomonas* sp. LV-5	100	EU580449	15.9	8.7	9.9	3.8	8.6	7.5
30	*Pseudomonas* sp. LV-2	98	EU580448	2.9	2.4	4.8	6.4	4.3	4.1
9	*Pseudomonas* sp. 65/3	100	EF513622	24.5	9.7	22.2	11.0	15.8	11.9
4	Gamma *Proteobacterium* RBE2CD-129	100	EF111261	5.9	7.6	16.2	11.9	12.5	8.7
	Unclassified bacteria								
2	Uncultured alpha *Proteobacterium*	85	AM237257	5.6		7.1		3.6	4.3
Stressed fish									
Band no	**Bacteroides**								
3	*Flavobacterium psychrolimnae*	99	AJ585428	1.8	4.1		6.3	5.2	3.9
8	*Flavobacterium* sp. SOC A4(51)	98	DQ628945	9.4	9.3	2.4	4.5	5.8	6.3
29	*Flavobacterium* sp. SOC A4(51)	100	DQ628945	8.9	6.7	4.5	6.3	7.3	4.6
	Firmicutes								
5	*Lactobacillus aviarius* subsp. *aviarius*	98	AB326355		9.5	1.0	6.9	3.9	9.5
17	*Lactobacillus aviarius*	98	AB175728		11.5		16.2	14.4	16.4
18	*Lactobacillus aviarius* subsp. *aviarius*	100	AB326355		7.9		7.2	13.9	11.4
19	*Lactobacillus letivazi*	95	AJ417738		5.2		13.8	4.7	7.5

Table 3. Contd.

11	*Anoxybacillus* sp.	96	DQ452025		4.8				
6	Uncultured *Lachnospiraceae* bacterium	99	EF705084	5.3					
	Proteobacteria								
7	*Vibrio* sp. B5-8a	100	DQ357798			9.7			
13	*Vibrio* sp. B5-8a	100	DQ357798			9.5			
16	*Vibrio* sp. BC-R3	98	DQ357806			18.4			
10	*Vibrio logei* strain T21110	97	DQ318955		2.3				
14	*Vibrio logei* strain T21110	99	DQ318955			8.6			
15	*Vibrio logei* strain T21110	98	DQ318955			6.3			
1	*Pseudomonas* sp. LV-5	100	EU580449	6.5	7.1	6.6	8.4	8.5	3.4
30	*Pseudomonas* sp. LV-2	98	EU580448	12.3	5.3	3.7	4.7	8.1	5.9
9	*Pseudomonas* sp. 65/3	100	EF513622	14.5	13.5	4.2	13.2	16.2	13.6
28	Uncultured *Pseudomonas* sp.	100	DQ189764				4.3	6.7	6.1
24	Uncultured *Photobacterium* sp.	100	AM936532			3.4			
4	Gamma *Proteobacterium* RBE2CD-129	100	EF111261	12.6	7.6	6.2	3.7	2.7	4.8
	Unclassified bacteria								
2	Uncultured alpha *Proteobacterium*	85	AM237257	8.1	5.6	3.5	4.5	4.5	7.3

Table 4. Growth inhibition of *Aeromonas salmonicida* LFI 4038 and *Moritella viscosa* LFI 5000 by carnobacteria isolated from three different regions of the GI tract of Atlantic salmon; prior to stress, 5 and 24 h after handling stress, and after 11 days of regular handling stress. After Ringø (unpublished data). The growth inhibition test was carried out using the microtitre method described by Ringø et al. (2005), Salma et al. (2011) and Askarian et al. (2012).

Parameter	***Aeromonas salmonicida* LFI 4038**				***Moritella viscosa* LFI 5000**			
	A	**B**	**C**	**Sum**	**A**	**B**	**C**	**Sum**
Ultimately before stress	17/17	8/9	12/12	37/38	4/17	1/9	1/12	6/38
5 h after stress	16/20	16/20	14/20	46/60	3/20	4/20	2/20	9/60
24 h after stress	11/21	13/20	16/23	30/64	1/21	3/20	1/23	5/64
11 days of regular stress	6/9	5/13	5/15	16/37	0/9	1/13	0/15	1/37
No. of carnobacteria able to inhibit the pathogen	50	42	47	139/199	8	9	4	21/199

Aeromonas salmonicida LFI 4038 was originally isolated from Atlantic salmon infected with furunculosis. *Moritella viscosa* LFI 5000 was originally isolated from Atlantic salmon from a natural outbreak of winter ulcer (NIFA, Tromsø, unpublished data). A - proximal intestine; B - mid intestine; C - distal intestine.

THE EFFECT OF ACUTE STRESS ON GUT MICROBIOTA IN ATLANTIC COD

Olsen et al. (2008) evaluated the effect of acute stress; similar to that used in the salmonid studies, in Atlantic cod. In general, the population level of adherent (autochthonous) cultivable bacteria (aerobic and facultative aerobic) ranged between 5.4 and 5.9 (log CFU g^{-1}) in cod intestines of undisturbed fish. Stress had no effect on the adherent bacterial population level in MI, but appeared to cause a small decrease, but non-significant, in both the hindgut (HG) and hindgut chamber (HC) sections. The fish did however seem to recover well, and by 48 h post stress there was a trend towards increased population level of cultivable gut bacteria in relation to initial levels in all groups (significant only for MI). The level of cultivable bacteria (log CFU) associated with digesta (allochthonous bacteria) from MI, HG and HC were 5.6, 5.6 and 5.9 CFU g^{-1}, respectively in undisturbed fish. A minor reduction was observed in MI 4 h after stress (log TVC = 5.1), but not revealed in the other sections. However, 48 h post stress, there was an increase in cultivable allochthonous bacteria in HG and HC with the most pronounced effect observed in HC (log 6.7 CFU g^{-1}). A total of 360 cultivable bacterial strains were isolated from the GI segments, before and 48 h post stress. Bacteria belonging to *Carnobacterium* dominated before stress and accounted for 63.3% of the cultivable bacteria. Of these were 22 strains identified as *Carnobacterium maltaromaticum* by partial sequence of 16S rRNA gene. However, subjecting Atlantic cod to stress reduced the proportion of *Carnobacterium* to approximately 23% of total cultivable bacteria. These data show many similarities to the salmonid studies referred above as the protective bacteria are eliminated following stress. This result might have relevance in the potential to retard pathogenic colonisation of the epithelial mucus layer within the intestine of fish, which is important because adhesion to epithelial mucus is thought to be an important step in intestinal infection, providing a foundation for mucosal interaction and translocation (Ringø, 2004; Ringø et al., 2010; Sica et al., 2012; Merrifield et al., 2014).

Isolates belonging to *Photobacterium phosphoreum* and *Photobacterium* spp. - like isolates were detected as allochthonous in MI of unstressed fish, while in fish 48 h post stress similar bacteria were isolated from HG (allochthonous) and HC; as autochthonous bacteria. Adherent *Pantoea agglomerans* and *Pantoea* spp. - like isolates were detected only in the MI of fish; 48 h after stress. In MI of unstressed fish, *Vibrio logei* and *Vibrio* spp. - like strains were isolated, while strains showing high similarity (98%) to an uncultured *Vibrio* sp. clone C7 were isolated in HG of stressed fish. *Bacillus* spp. was detected associated with digesta from the HG of unstressed fish, but was not detected in stressed fish. *Enterobacter* was detected in the gut of stressed fish. In contrast, *Enterobacter* strains were not detected in any sample prior to stress. Differences between unstressed and stressed fish can further be illustrated by the fact that strains showing high similarity to uncultured bacterium clone E792 and uncultured bacterium clone AKIW6008 were only detected in the gut of unstressed fish, but then as allochthonous bacteria present in HG and HC.

The peel-off mucus effect observed in salmonids (Olsen et al., 2002; 2005) was not observed in the Atlantic cod study and these results were supported by Ussing chamber data from four fish per treatment where the transepithelial resistance increased with time suggesting a higher mucus production and that this mucus is attached to the intestinal mucosa (Olsen et al., 2008).

PROBIOTICS AND STRESS

Currently there is a growing interest in the use of probiotics in aquaculture (Lauzon et al., 2014; Newaj-Fyzul et al., 2013). Mohapatra et al. (2013) discussed different aspects of stress management and probiotic intervention, but as no information is reported on the role of probiotics in fish related to different environmental stress and their effect on gut microbiota and fish health, we recommend that the topic should be given high priority in the future.

When discussing the use of probiotics in aquaculture and the antagonistic effect of gut bacteria towards pathogens, it is worth to mention that in a probiotic study with Atlantic salmon, Gram et al. (2001) used *Pseudomonas fluorescens* strain AH2; a strain showing strong *in vitro* inhibitory activity towards *A. salmonicida* (Gram et al., 1999). However, co-habitant infection by *A. salmonicida* in Atlantic salmon did not result in any effect on furunculosis-related mortality (Gram et al., 2001). Based on their results, the authors concluded that a strong *in vitro* growth inhibition cannot be used to predict a possible *in vivo* effect.

CONCLUSIONS AND FURTHER PERSPECTIVES

One of the most important goals for fish microbiologists has been to obtain a stabile indigenous gut microbiota of fish. However, hierarchy formation, acute stress and handling stress, the microbial balance become disturbed and disordered, and the complex relationship of the indigenous gut microbiota with the host can be depicted in a multilevel framework; where luminal bacteria, the mucus layer and the innate and adaptive immunity interact.

The practical effect of this activity is the exclusion of beneficial bacteria which may result in adherence and colonisation of invading populations of nonindigenous microorganisms, including pathogens in the GI tract in fish. The antagonistic effect towards pathogens by the

gut microbiota is possibly mediated by competition for nutrients and adhesion sites, formation of metabolites such as organic acids, hydrogen peroxide, and production of antibiotic-like compounds and bacteriocins. A fundamental question arises when discussing the protective role of the GI microbiota; is the antagonistic gut microbiota affected by stress and do changes in the gut microbiota have any negative health effect? This has to be elucidated in future studies.

REFERENCES

Asfie M, Yoshijima T, Sugita H (2003). Characterization of the goldfish fecal microflora by the fluorescent in situ hybridization method. Fish Sci. 69(1):21-26.

Askarian F, Zhou Z, Olsen RE, Sperstad S, Ringø E (2012). Culturable autochthonous gut bacteria in Atlantic salmon (*Salmo salar* L.) fed diets with or without chitin. Characterization by 16S rRNA gene sequencing, ability to produce enzymes and *in vitro* growth inhibition of for fish pathogens. Aquaculture 326-329:1-8.

Birkbeck TH, Ringø E (2005). Pathogenesis and the gastrointestinal tract of growing fish. In Microbial Ecology in Growing Animals, Holzapfel W, Naughton P (eds.). Elsevier, Edinburgh, UK. pp. 208-234.

Biron M, Benfey TJ (1994). Cortisol, glucose and haematocrit changes during acute stress, cohort sampling, and the diel cycle in diploid and triploid brook trout (*Salvelinus fontinalis* Mitchill). Fish Physiol. Biochem. 13(2):153-160.

Gatesoupe FJ (1999). The use of probiotics in aquaculture. Aquacult. 180(1-2):147-165.

Gomez GD, Balcázar JL (2008). A review on the interactions between gut microbiota and innate immunity of fish. FEMS Immunol, Med. Microbiol. 52(2):145-154.

Gram L, Løvold T, Nielsen J, Melchiorsen J, Spanggaard B (2001). *In vitro* antagonism of the probiont *Pseudomonas fluorescens* strain AH2 against *Aeromonas salmonicida* does not confer protection of salmon against furunculosis. Aquaculture 199(1-2):1-11.

Gram L, Melchiorsen J, Spanggaard B, Huber I, Nielsen TF (1999). Inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens* strain AH2, a possible probiotic treatment of fish. Appl. Environ. Microbiol. 65(3):969-973.

Groff J, LaPatra S (2000). Infectious diseases impacting the commercial culture of salmonids. J. Appl. Aquacult. 10(4): 17-90.

Hoskonen P, Pirhonen J (2006). Effects of repeated handling, with or without anaesthesia, on feed intake and growth in juvenile rainbow trout, *Oncorhynchus mykiss* (Walbaum). Aquacult. Res. 37(4):409-415.

Jöborn A, Dorsch M, Olsson JC, Westerdahl A, Kjelleberg S (1999). *Carnobacterium inhibens* sp. nov., isolated from intestine of Atlantic salmon (*Salmo salar*). Int. J. Sys. Bacteriol. 49(4):1891-1898.

Jutfelt F, Olsen RE, Glette J, Ringø E, Sundell K (2006). Translocation of viable *Aeromonas salmonicida* across the intestine of rainbow trout. J. Fish Dis. 29(5):255-262.

Lauzon HL, Pérez-Sánchez P, Merrifield DL, Ringø E, Balcázar L (2014). Probiotic applications in coldwater fish species. In Probiotics, Prebiotics and Gut Health in Aquaculture Nutrition, Merrifield D, Ringø E (eds.), in press. Wiley-Blackwell Publishing, Oxford, UK.

Liu Y, Zhou Z, Yao B, Shi P, He S, Hølvold LB, Ringø E (2008). Effect of intraperitoneal injection of immunostimulatory substances on allochthonous gut microbiota of Atlantic salmon (*Salmo salar* L.) determined using denaturing gradient gel electrophoresis. Aquacult. Res. 39(6):635-646.

Lutgendorff F, Akkermans LMA, Söderholm JD (2008). The role of microbiota and probiotics in stress-induced gastrointestinal damage. Curr. Mol. Med. 8(4):282-298.

Mazeaud MM, Mazeaud F, Donaldson EM (1977) Primary and secondary effects of stress in fish - some new data with a general review. Trans. Am. Fish Soc. 106(3):201-212.

Merrifield DL, Balcazar JL, Daniels CL, Zhou Z, Carnevali O, Sun YZ, Hoseinifar SH, Ringø E (2014). Indigenous lactic acid bacteria in fish and crustaceans. In Probiotics, Prebiotics and Gut Health in Aquaculture Nutrition, Merrifield D, Ringø E (eds.), in press. Wiley-Blackwell Publishing, Oxford, UK.

Mohapatra S, Chakraborty T, Kumar V, deBoeck G, Mohanta KN (2013). Aquaculture and stress management: a review of probiotic intervention. J. Anim. Physiol. Anim. Nutr. 97(3):405-430.

Muroga K, Higashi M, Keitoku H (1987). The isolation of intestinal microflora of farmed seabream (*Pagrus major*) and black seabream (*Acanthopagrus schlegeli*) at larval and juvenile stages. Aquaculture 65(1):79-88.

Nayak SK (2010). Role of gastrointestinal microbiota in fish. Aquacult. Res. 41(11):1553-1573.

Newaj-Fyzul A, Al-Harbi AH, Austin (2013). Review: developments in the use of probiotics for disease control in aquaculture. Aquacult. early view, doi: 10.1016/j.aquaculture.2013.08.026

Olsen RE, Sundell K, Hansen T, Hemre G-I, Myklebust R, Mayhew TM, Ringø E (2002). Acute stress alters the intestinal lining of Atlantic salmon, *Salmo salar* L.: An electron microscopical study. Fish Physiol. Biochem. 26(3):211-221.

Olsen RE, Sundell K, Mayhew TM, Myklebust R, Ringø E (2005). Acute stress alters intestinal function of rainbow trout, *Oncorhynchus mykiss* (Walbaum). Aquacult. 250(1-2):480-495.

Olsen RE, Sundell K, Ringø E, Myklebust R, Hemre G-I, Hansen T, Karlsen Ø (2008). The acute stress response in fed and food deprived Atlantic cod, *Gadus morhua* L. Aquacult. 280(1-4):232-241.

Paull GC, Filby AL, Giddins HG, Coe TS, Hamilton PB, Tyler CR (2010). Dominance hierarchies in zebrafish (*Danio rerio*) and their relationship with reproductive success. Zebrafish 7(1):109-117.

Peters G (1982). The effect of stress on the stomach of European eel, *Anguilla anguilla* L. J. Fish Biol. 21(5): 497-512.

Peters G, Faisal M, Lang T, Ahmed I (1988). Stress caused by social interactions and its effect on susceptibility to *Aeromonas hydrophila* infection in rainbow trout *Salmo gairdneri*. Dis. Aquatic Org. 4(2):83-89.

Pickering AD, Pottinger TG (1989). Stress responses and disease resistance in salmonid fish: Effects of chronic elevation of plasma cortisol. Fish Physiol. Biochem. 7(1-4):253-258.

Qiao Y, Sun J, Ding Y, Le G, Shi Y (2013). Alterations of the gut microbiota in high-fat diet mice is strongly linked to oxidative stress. Appl. Microbiol. Biotechnol. 97(4):1689-1697.

Ringø E (1993). The effect of chromic oxide (Cr_2O_3) on aerobic bacterial populations associated with the epithelial mucosa of Arctic charr, *Salvelinus alpinus* (L.). Can. J. Microbiol. 39(12):1169-1173.

Ringø E (2004). Lactic acid bacteria in fish and fish farming. In Lactic Acid Bacteria, Salminen S, Ouwehand A, von Wrigth A (eds.). Marcel Dekker Inc., New York. pp. 581-610.

Ringø E (2008). The ability of carnobacteria isolated from fish intestine to inhibit growth of fish pathogenic bacteria: a screening study. Aquacult. Res. 39(2):171-180.

Ringø E, Bendiksen HR, Wesmajervi MS, Olsen RE, Jansen PA, Mikkelsen H (2000). Lactic acid bacteria associated with the digestive tract of Atlantic salmon (*Salmo salar* L.). J. Appl. Microbiol. 89(2):317-322.

Ringø E, Jutfelt F, Kanapathippillai P, Bakken Y, Sundell K, Glette J, Mayhew TM, Myklebust R, Olsen RE (2004). Damaging effect of the fish pathogen *Aeromonas salmonicida* ssp. *salmonicida* on intestinal enterocytes of Atlantic salmon (*Salmo salar* L.). Cell Tiss. Res. 318(2):305-311.

Ringø E, Lødemel JB, Myklebust R, Kaino T, Mayhew TM, Olsen RE (2001). Epithelium associated microbes in the gastrointestinal tract of Arctic charr (*Salvelinus alpinus* L.). An electron microscopical study. J. Appl. Microbiol. 90(2):294-300.

Ringø E, Løvmo L, Kristiansen M, Bakken Y, Salinas I, Myklebust R, Olsen RE, Mayhew TM (2010). Lactic acid bacteria *vs*. pathogens in the gastrointestinal tract of fish: a review. Aquacult. Res. 41(4):451-467.

Ringø E, Olsen RE (1999). The effect of diet on aerobic bacterial flora associated with intestine of Arctic charr (*Salvelinus alpinus* L.). J. Appl. Microbiol., 86(1):22-28.

Ringø E, Olsen RE, Øverli Ø, Løvik F (1997) Effect of dominance hierarchy formation on aerobic microbiota associated with epithelial

mucosa of subordinate and dominant individuals of Arctic charr, *Salvelinus alpinus* (L.). Aquacult. Res. 28(11):901-904.
Ringø E, Schillinger U, Holzapfel W (2005). Antibacterial abilities of lactic acid bacteria isolated from aquatic animals and the use of lactic acid bacteria in aquaculture. In Microbial Ecology in Growing Animals, Holzapfel W, Naughton P (eds.). Elsevier, Edinburgh, UK. pp. 418-453.
Rollo A, Sulpizio R, Nardi M, Silvi S, Orpianesi C, Caggiano M, Cresci A, Carnevali O (2006). Live feed supplement in aquaculture for improvement of stress tolerance. Fish Physiol. Biochem. 32(2):167-177.
Salma W, Zhou Z, Wang W, Askarian F, Kousha A, Ebrahimi MT, Myklebust R, Ringø E (2011). Histological and bacteriological changes in intestine of beluga (*Huso huso*) following *ex vivo* exposure to bacterial strains. Aquaculture 314(1-4):24-33.
Sandodden R, Finstad B, Iversen M (2001). Transport stress in Atlantic salmon (*Salmo salar* L.): anaesthesia and recovery. Aquacult. Res. 32(2):87-90.
Shepard KL (1994). Functions of fish mucus. Rev. Fish Biol. Fish. 4(4):401-429.
Sica MG, Brugnoni LI, Marucci PL, Cubitto MA (2012). Characterization of probiotic properties of lactic acid bacteria isolated from an estuarine environment for application in rainbow trout (*Oncorhynchus mykiss*, Walbaum) farming. Ant. Leeuwenh. Int. J. Gen. Mol. Microbiol. 101(4):869-879.
Sneddon LU, Margareto J, Cossins AR (2005). The use of transcriptomics to address questions in behaviour: production of a suppression subtractive hybridisation library from dominance hierarchies of rainbow trout. Physiol. Biochem. Zool. 78(5):695-705.
Snieszko SF (1974). The effects of environmental stress on out-breaks of infectious diseases in fish. J. Fish Biol. 6(2):197-208.
Sugita H, Okano R, Suzuki Y, Iwai D, Mizukami M, Akiyama N,. Matsuura S (2002). Antibacterial abilities of intestinal bacteria from larval and juvenile Japanese flounder against pathogens. Fish Sci. 68(5):1004-1011.
Szakolczai J (1997). Histopathological changes induced by environmental stress in common carp, Japanese coloured carp, European eel, and African catfish. Acta Vet. Hung. 45(1):1-10.
Tannock GW, Savage DC (1974). Influences of dietary and environmental stress on microbial populations in the murine gastrointestinal tract. Infect. Immunol. 9(3):591-598.
Torrecillas S, Makol A, Caballero MJ, Montero D, Dhanasiri AKS, Sweetman J, Izquierdo M (2012). Effects on mortality and stress response in European sea bass, *Dicentrarchus labrax* (L.), fed mannan oligosaccharides (MOS) after *Vibrio anguillarum* exposure. J. Fish Dis. 35(8):591-602.
Van Ham EH, Van Anholt RD, Kruitwagen G, Imsland AK, Foss A, Sveinsbø BO, Fitzgerald R, Parapoura AC, Stefansson SO, Bonga, SEW (2003). Environment affects stress in exercised turbot. Comp. Biochem. Physiol. A 136(3):525-538.
Wedemeyer G (1970). The role of stress in the disease resistance in fish. Spec. Pub. Am. Fish. Soc. 5:30-35.

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Full Length Research Paper

Determination of multiple antibiotic resistance patterns and indexing among metal tolerant β-lactamase-producing *Escherichia coli*

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The antibiotic resistance profiles of *Escherichia coli* isolated from three different sampling sites of the Gomti River at Lucknow city was evaluated. Water samples were collected and then analyzed for the presence of *E. coli*, using standard methods. Antibiotic susceptibility testing was performed by the disc diffusion method. Of the 77 *E. coli* isolates tested, marked antibiotic resistances (over 70%) were observed for amoxicillin, nitrofurozone, chloramphenicol, polymixin B, methicilin, ampicillin, nalidixic acid, cefpodoxime, erythromycin, penicillin, rifampicin and ofloxacin depending upon the sampling sites. All *E. coli* isolates also showed multiple resistance patterns in different combination of antibiotics. The MAR index ranges were found very high indicating the high risk of environmental contamination. The findings indicated that pollution of aquatic environments from different sources of the city may have a potential impact on the dissemination and survival of *E. coli*, as well as other pathogenic bacteria in the Gomti River water for public and animal health. This may result to a negative effect on antibiotic therapy for infectious diseases.

Key words: Gomti River, coliforms, antibiotic susceptibility, multiple antibiotic resistance (M.A.R).

INTRODUCTION

Many antibiotics have been used in the last several years in medical, veterinary, agriculture and aquaculture practices (Alpay-Karaoglu et al., 2007). Recently, there has been a growing interest in the presence of different pharmaceutical substances, mainly antibiotics in the aquatic environment (Vulliet and Cren-Olive, 2011). The wide application of antibiotics by human has led to large-scale dissemination of bacteria resistant to antibiotics in water basins (Dang et al., 2006).

Bacterial resistance to antibiotics in the aquatic environment has received comparatively little attention. Bacterial contamination of surface water, particularly contaminated with faecally derived bacteria, has long been a water quality issue due to the potential for disease transmission. Because of this and the potential for antibiotic resistance, there is a new level of risk associated with these bacteria. Recent studies have also identified antibiotics themselves in surface waters (Batt et al., 2006) and the role of these antibiotics in the development, transfer and maintenance of resistance is largely unknown. The number of antimicrobial-resistant (AMR) bacteria in the environment increases exponentially with the use of

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Abbreviations: MAR, Multiple antibiotic resistance; **AMR,** antimicrobial-resistant; **IMViC tests,** indole, methyl red, Voges Proskauer and citrate utilization tests.

antimicrobials, as a result of increasing selective pressure on bacterial populations (Tsiodras et al., 2008). Furthermore, AMR is increasing, and its spread between different bacterial strains in different habitats has been demonstrated (Drewnowska and Swiecicka, 2013; SVARM, 2006).

Untreated drinking water coming from different sources contains coliforms including *Esherichia coli*. In developing countries, drinking water supply lines and open sewage drains are laid side by side resulting in frequent contamination of water (Patoli et al., 2010). *E. coli* is an opportunistic pathogen. The disposal of treated sewage into rivers, lakes, or elsewhere may or may not influence environmental bacterial populations (Ahmed et al., 2010). Food and water borne outbreaks of *E. coli* have been documented from a number of countries (Soderstrom et al., 2008). Increase in antibiotic resistance level is now a global problem. Since water is one of the four components of environment, and a usual habitat for *E. coli*, therefore, the availability of antibiotic resistant *E. coli* strains in water cannot be denied.

They represent one of the major contaminants in surface and ground water in developing countries. In recent decades, the increased usage of antibiotics has led to antibiotic resistance among enteric bacteria. River water is the main receptacle reservoir of antibiotics and antibiotic resistant bacteria in the environment. They are directly introduced into surface water through animal farms and agricultural practices. The antibiotic resistance bacteria in drinking water are a prime concern to public health (Igbinosa and Okoh, 2008). The wide use and abuse of antibiotics in human therapy has produced MAR *E. coli* in the faeces of human as well (Fischbach and Walsh, 2009). These practices have resulted in the coexistence of MAR *E.coli* within these major reservoirs of enteric disease for human. The aim of this study was to investigate the multiple antibiotic resistance and their patterns among the beta lactamase producing *E. coli* strains from the Gomti river water in the vicinity of Lucknow city.

MATERIALS AND METHODS

Sampling

The study was carried out on the Gomti River water of Lucknow City. Water samples were collected from three different sampling sites in sterile 250 ml polypropylene bottles, according to STAS 3001-91. Samples were taken at 4°C until their arrival in laboratory. This study was undertaken to determine the incidence and antibiotic resistant patterns of *E. coli* strains isolated from water samples. 77 *E. coli* isolates were isolated and tested against 20 commonly used antimicrobial agents.

Isolation and identification of metal tolerant *E. coli* isolates

Isolation of metal tolerant *E. coli* isolates from water samples were done on metal (Cr, Cd, Co, Cu, Zn, Ni and Hg) amended EMB agar plates at 100 µg/ml concentration. Serial dilutions of the water samples were plated by spreading 0.1 ml on EMB medium for metal tolerant *E. coli*. Plates were incubated at 37°C for 24 h. Greenish with metallic sheen colonies were identified as *E. coli* and further characterization was done by indole, methyl red, Voges Proskauer and citrate utilization tests (IMViC tests).

Beta lactamase production

For detection of beta lactamase producing bacteria, a loopfull of grown culture was transferred into small tube containing 1 ml of penicillin G solution and incubated at 37°C for 30 min. 0.5 ml of iodine solution was added and mixed for 2-3 min. Change in colour to colourless, indicates positive result (Dierikx et al., 2010)

Determination of antibiotic resistance

The antibiotic resistance was determined by a standard disc diffusion technique using Mueller-Hinton agar (Difco) according to the recommendations of National Committee for Clinical Laboratory Standards (NCCLS 2008) including *Escherichia coli* ATCC 25922 as a control strain. The antimicrobial drugs tested and their sensidisk concentrations were: Amoxicillin (AMX) 25 µg, Nalidixic acid (NA) 30 µg, Neomycin (NEO) 30 µg, Kanamycin (KAN) 30 µg, Ampicillin (AMP) 10 µg, Cefradine (CEF) 25 µg, Gentamycin (GEN) 30 µg, Nitrofurozone (NR) 100 µg, Chloramphenicol (CHMP) 30 µg, Polymixin B (PB) 300 µg, Methicillin (MET) 5 µg, Streptomycin (STREPTO) 25 µg, Penicillin (PEN) 10 µg, Cefpodoxime (CPD) 10 µg, Rifampicin (RIF) 2 µg, Ciprofloxacin (CIP) 5 µg, Erythromycin (ERYTHRO) 15 µg, Ofloxacin (OF) 2 µg, Sulphadiazine (SZ) 300 µg and Tetracycline (TET) 10 µg. Within 15 min of the application of the discs, the plates were inverted and incubated at 37°C. After 24 h of incubation, the plates were examined, and the diameters of the zones of complete inhibition to the nearest whole millimetre were measured. The zone diameter for individual antimicrobial agents was then translated into sensitive and resistant categories. These antimicrobial agents were chosen based on their importance in treating human or animal *E. coli* infections and their use as feed additives to promote growth in animals in agriculture, zootechny and aquaculture (Florea, 2011)

Multiple antibiotic resistances (MAR) indexing

The MAR index profile based on isolate and sampling site was performed to evaluate the health risk of the environment. MAR index for test isolates was calculated according to the formula: No. of antibiotics to which all isolates were resistant/No. of antibiotics tested x No. of isolates as recommended by Downing et al. (2011). Sampling site based MAR index was calculated by the same formula modified by the total number of isolates from a sampling site as described (Riaz et al., 2011).

RESULTS

A total of 77 *E. coli* isolates were isolated from three different sites, 27 from site I and 25 each from sites II and III of the Gomti River Water. All the isolates were found beta lactamase positive and characterized on the basis of antibiotic susceptibility test. *E. coli* isolates from site I, II and III showed a variable resistance against 20 different antibiotics tested showed in Figure 1. 96% of the isolates showed resistance against amoxicillin followed by 89, 85 and 77% against Nitrofurozone, Penicillin, Chloramphenicol, respectively. Lower number of isolates showed resistance against Rifampicin (14.8%) followed by Gentamicin (11.11%), (Neo 7.40%) and ciprofloxacin (3.7%).

In case of site II: A high level of resistance was also observed among the isolates; all the isolates demonstrated resistance against Amoxicillin, Polymixin B and Methicilin.

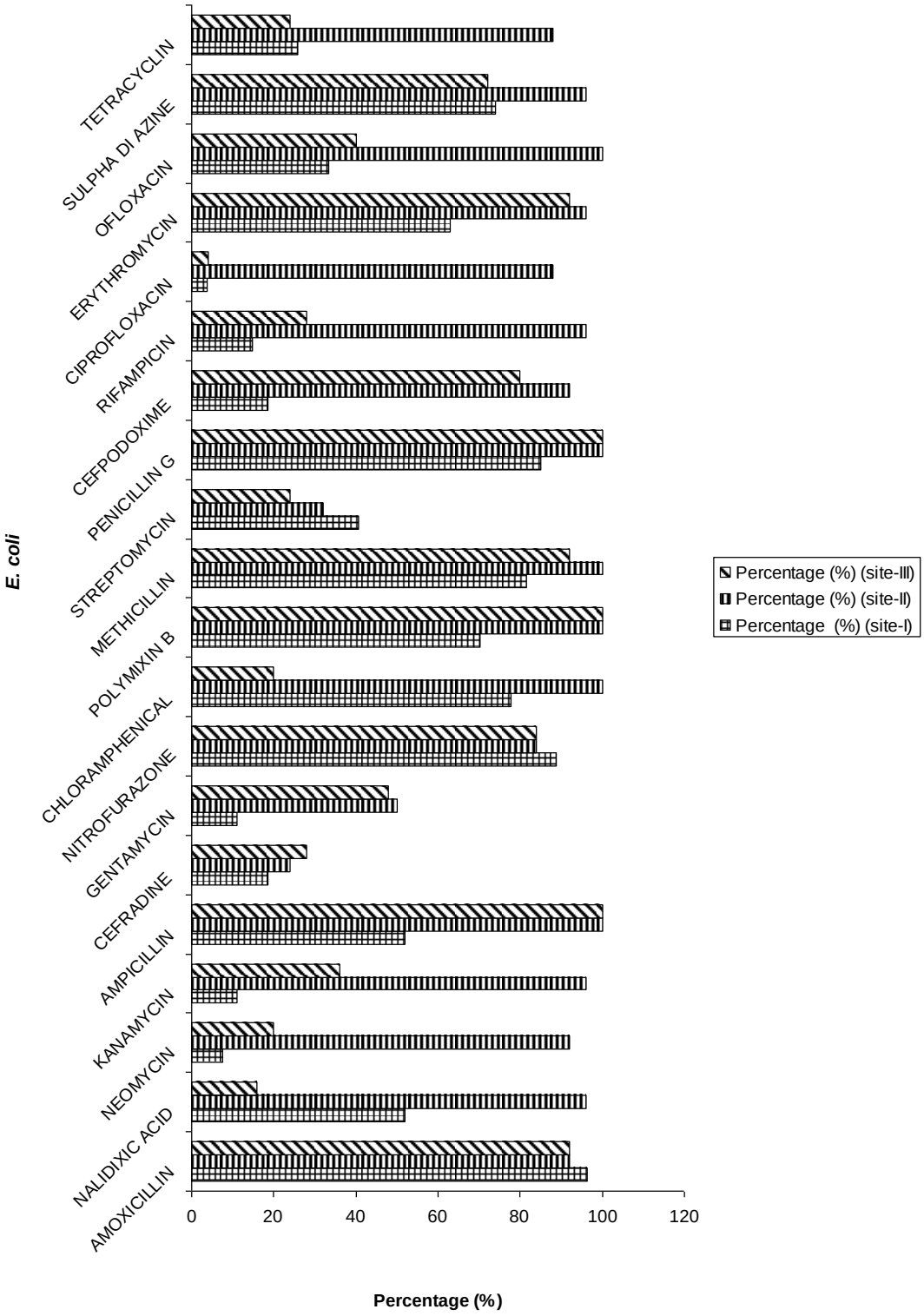

Figure 1. Percentage of resistance against antibiotics in *E. coli* isolates from Gomti River water (sites I, II and III).

96% of the isolates showed resistance against cefpodoxime, rifampicin and ciprofloxacin. Minimum drug resistance was observed by 40, 36, 32% isolates against Kanamycin, Tetracyclin and Gentamicin, respectively.

Similar observations were recorded in the case of site III: All isolates demonstrated resistance against Ampicillin, Polymixin B and Penicillin, while 92 and 84% isolates showed resistance against Amoxicillin and Nitrofurozone,

Table 1. Antibiotic resistance pattern in 27 *E. coli* isolates from Gomti River water (sample 1).

Number of antibiotics	Resistance pattern	Number of resistance isolates	Percentage (%)	MAR
2	AMX, MET.	1	3.7	0.1
4	AMX, AMP, ERYTHRO, PB.	1	3.7	0.2
5	PEN, ERYTHRO, NEO, PB, AMX	1	3.7	0.25
6	NR, NA, AMX, OF, RIF, PB. NR, MET, AMOX, PB, RIF, SZ.	2	7.4	0.3
8	AMX, NA, NR, CHMP, PEN, ERYTHRO, SZ, OF. AMX, NR, MET, PEN, ERYTHRO, SZ, KAN, CHMP. NR, CHMP, MET, PEN, ERYTHRO, SZ, AMP, TET.	3	11.1%	0.4
9	AMX, NR, CHMP, MET, STREPTO, PEN, SZ, AMP, PB. AMX, NR, CHMP, MET, STREPTO, SZ, PEN, PB, AMP. AMX, NR, MET, STREPTO, PEN, SZ, CHMP, TET, OF.	4	14.8%	0.45
10	AMX, NA, NEO, GEN, NR, ERYTHRO, SZ, CHMP, PB. AMX, NA, NR, MET, PEN, CPD, ERYTHRO, SZ, PB, CHMP. MET, CHMP, NR, NA, AMOX, PEN, ERYTHRO, TET, CPD, PB. NA, NR, MET, PEN, CPD, ERYTHRO, CHMP, AMOX, AMP, PB. AMX, NR, CH, MET, STREPTO, PEN, SZ, AMP, PB, RIF. AMX, NA, NR , MET, STREPTO, PEN, SZ, CEF, CHMP, NEO. AMX, NA, AMP, NR, CHMP, MET, PEN, ERYTHRO, SZ, PB. AMX, NR, CHMP, PB, MET, PEN, ERYTHRO, SZ, TET, KAN. AMX, AMP, GEN, NR, CHMP, MET, PEN, ERYTHRO, OF, SZ.	8	25.6%	0.5
11	AMX, NA, NR, MET, STREPTO, PEN, CPD, SZ, ERYTHRO, OF, PB. AMOX, NA, CEF, NR, CHMP, MET, PEN, CPD, ERYTHRO, OF, AMP. AMX, NA, NR, CHMP, MET, STREPTO, PEN, SZ, AMP, PB, OF. AMX, NR, MET, STREPTO, PEN, SZ, CHMP, TET, PB, AMP, CEF. AMX, NA, NR, MET, STREPTO, PEN, SZ, CH, PB, AMP, OF.	5	18.5%	0.55
12	AMX, NA, NR, CHMP, MET, STREPTO, PEN, SZ, AMP, PB, OF, ERYTHRO.	1	3.7	0.6
14	AMX, NA, CEF, GEN, NR, CHMP, PB, MET, PEN, RIF, CIP, ERYTHRO, SZ, AMP.	1	3.7	0.7

Sensitive strains- 2.

respectively. There in site III, the least number of isolates by 16 and 4% showed resistance against Nalidixic acid and Ciprofloxacin, respectively.

Single and multiple antibiotic resistance patterns in 27 *E. coli* isolates were also recorded in the sites of the Gomti River Water, as depicted in Tables 1, 2 and 3. In site I, all the isolates showed 11 patterns of antibiotic resistance against the antibiotics tested. 3.7% isolates showed resistance to 2, 4, 5, 12 and 14 antibiotics in one and two combinations, respectively. 7.4% isolates showed resistance to 6 antibiotics at a time in 2 different combinations. 11.1% isolates showed resistance to 8 antibiotics at a time in three different combinations. 14% of the isolates exhibited resistance to 9 antibiotics at a time in four combinations. 18.5 and 29.6% of the isolates showed resistance to 11 and 10 antibiotics at a time in five and eight different combinations, respectively.

Antibiotic resistance patterns among the 25 *E. coli* isolates from site II were also recorded. All the isolates showed 12 different resistance patterns among the antibiotics tested. 4% isolates showed resistance to 6, 8, 9, 10 and 14 antibiotics at a time in one combination and 8% isolates exhibited resistance to 12, 13, 16 and 18 antibiotics at a time in two different combinations and 12, 16 and 20% isolates showed resistance to 17, 11and 15 antibiotics at a time in three, four and five different combinations, respectively.

In the case of site III (25 *E. coli* isolates), all the isolates showed 12 different patterns of antibiotic resistance against the antibiotics tested. 4% of the isolates showed resistance to 7, 8, 11, 18 and 20 antibiotics at a time in one combination, respectively, while 8% of the isolates exhibited resistance to 10, 12, 14, 15 and 17 antibiotics at a time in two different combinations respectively. 16 and 24% of *E. coli* isolates exhibited resistance to 16 and 13 antibiotics at a time in four and six different combinations, respectively.

M.A.R. indexing based on isolates was also calculated. A varied trend of MAR Index was observed among the isolates from the three different sampling sites. 3.7%

Table 2. Antibiotic resistance pattern in 25 *E. coli* isolates from the effluent of Gomati River water (sample 2).

Number of antibiotics	Resistance pattern	Number of resistance isolates	Percentage (%)	MAR
6.	AMX, PEN, SZ, CPD, MET, PB.	1	4	0.3
8.	CPD, MET, NA, PB, GEN, RIF, AMX, NR.	1	4	0.4
9.	PB, CPD, OF, GEN, RIF, AMX, PEN, STREPTO, MET.	1	4	0.45
10.	AMX, PEN, MET, PB, AMP, OF, KAN, CHMP, RIF, ERYTHRO.	1	4	0.5
11.	AMX, PEN, MET, NA, PB, AMP, CHMP, ERYTHRO, RIF, NEO, OF, PB, AMX, CPD, MET, NR, NA, NEO, PEN, OF, RIF, ERYTHRO. CPD, MET, CHMP, NR, CEF, SZ, PB, PEN, ERYTHRO, AMOX, RIF. AMX, PEN, CPD, MET, CH, NA, PB, AMP, STREPTO, OF, RIF.	4	16	0.55
12.	AMX, PEN, CPD, MET, CHMP, NA, KAN, STREPTO, RIF, OF, NEO, PB, PB, OF, AMX, PEN, MET, NA, GEN, STREPTO, KAN, AMP, RIF, CPD.	2	8	0.6
13.	AMX, PEN, SZ, CPD, MET, AMP, NA, STREPTO, CEF, NR, PB, RIF, ERYTHRO, SZ, CPD, MET, PB, AMX, PEN, NR, NA, ERYTHRO, NEO, RIF, OF, AMP.	2	8	0.05
14.	AMX, PEN, SZ, CPD, MET, AMP, CHMP, NA, STREPTO, ERYTHRO, RIF, PB, OF, CEF.	1	4	0.7
15.	AMX, PEN, MET, AMP, CH, NA, TET, RIF, KAN, CPD, PB, NEO, OF, SZ, ERYTHRO. AMX, PEN, SZ, CPD, MET, NR, NA, AMP, CIP, CEF, OF, CH, ERYTHRO, RIF, PB. OF, AMX, PEN, SZ, CPD, MET, AMP, CHMP, NA, ERYTHRO, KAN, RIF, PB, CIP, TET.	5	20	0.75
16.	AMX, PEN, CEF, SZ, MET, CHMP, NR, ERYTHRO, PB, OF, KAN, RIF, AMP, CPD, TET. CEF, SZ, CPD, MET, RIF, AMX, PEN, CHMP, CIP, PEN, NA, OF, AMP, TET, ERYTHRO. PEN, MET, AMP, CHMP, NA, RIF, AMX, OF, KAN, PB, CPD, TET, ERYTHRO, NEO, STREPTO, NR. AMP, PEN, CPD, MET, AMP, CHMP, NA, ERYTHRO, PB, GEN, KAN, RIF, SZ, OF, NR, NEO.	2	8	0.8
17.	PB, AMX, PEN, CEF, SZ, CPD, MET, AMP, CHMP, NR, NA, RIF, ERYTHRO, GEN, OF, NEO, CIP. RIF, AMX, OF, PEN, SZ, CPD, MET, AMP, CHMP, CIP, NA, TET, PB, GEN, KAN, NEO, NR.	3	12	0.85
18.	MET, CHMP, ERYTHRO, PB, OF, KAN, RIF, AMP, TET, NA, ERYTHRO, STREPTO, CHMP, PB, TET, NEO, OF. PB, PEN, SZ, CPD, MET, NR, CHMP, AMX, RIF, GEN, CEF, AMP, TET, NEO, CIP, NA, OF, KAN. AMX, PEN, CPD, MET, CIP, NA, ERYTHRO, PB, GEN, CEF, SZ, CHMP, STREPTO, NR, RIF, OF, NEO, AMP.	2	8	0.9

isolates from site I showed a MAR 0.1 - 0.7 range against different number of antibiotics. M.A.R. 0.45, 0.5 and 0.55 were recorded by 14.8, 29.6 and 18.5% isolates respectively. In the case of sampling site II, 4% isolates demonstrated 0.3 - 0.7 MAR. range, while, MAR 0.9 was recorded by 8% isolates against 18 antibiotics. Maximum MAR was recorded among the isolates from site III. 4 and 8% isolates showed MAR range 0.35 - 1.0 and 0.6 - 0.9 against different number of antibiotics, respectively.

DISCUSSION

Many studies revealed that the co-selection took place in antibiotic resistance (Berg et al., 2005; Stepanauskas et al., 2005; Wright et al., 2006). Bacteria in metal-contaminated environments appeared to be easier to obtain antibiotic resistance phenotypes than in control areas (Baker-Austin et al., 2006). Wright et al. (2008) found that class 1 integrase gene was more abundant in the metal-exposed

Table 3. Antibiotic resistance pattern in 25 *E. coli* isolates from the effluent of Gomati River water (sample 3).

Number of antibiotics	Resistance pattern	Number of resistance isolates	Percentage (%)	MAR
7.	CPD, AMX, PEN, CEF, AMP, PB, MET.	1	4	0.35
8.	CPD, AMX, MET, ERYTHRO, NR, PEN, PB, RIF	1	4	0.4
10.	CPD, PEN, MET, ERYTHRO, PB, AMX, KAN, CHMP, NR, AMP. CPD, SZ, OF, PEN, ERYTHRO, NR, CHMP, MET, PB, AMX.	2	8	0.5
11.	CPD, CEF, MET, KAN, AMX, PB, ERYTHRO, NEO, NA, AMP, OF.	1	4	0.55
12.	CPD, AMX, MET, NR, RIF, STREPTO, AMP, CHMP, PB, NA, CEF, PEN. CPD, PEN, CEF, CIP, AMP, MET, AMX, PB, SZ, OF, NA, NR	2	8	0.6
13.	CPD, SZ, AMX, PEN, CEF, NA, MET, ERYTHRO, NR, PB, RIF, CHMP, AMP. CPD, AMX, PEN, CHMP, MET, ERYTHRO, RIF, STREPTO, AMP, NR, OF, NA, NEO. CPD, AMX, PEN, CEF, AMP, MET, RIF, PB, CHMP, NA, ERYTHRO, NR, KAN. CPD, AMX, PEN, AMP, MET, PB, TET, NA, ERYTHRO, NR, KAN, GEN. CPD, AMX, PEN, CEF, MET, NR, ERYTHRO, AMP, OF, CIP, NA, PB, SZ. CPD, AMX, PEN, CEF, TET, OF, ERYTHRO, PB, KAN, MET, AMP, NR, NA.	6	24	0.65
14.	CPD, PEN, MET, ERYTHRO, RIF, CHMP, TET, CIP, AMX, PB, KAN, NR, NEO, AMP. CPD, SZ, AMX, CEF, NA, AMP, MET, NR, RIF, TET, OF, PEN, PB, KAN.	2	8	0.7
15.	CPD, SZ, AMX, PEN, CEF, TET, CHMP, MET, RIF, OF, NA, PB, AMP, GEN, NR. CPD, AMX, PEN, CEF, MET, NEO, PB, SZ, ERYTHRO, NR, OF, NA, AMP, TET, KAN.	2	8	0.75
16.	CPD, AMX, PEN, NA, CHMP, MET, NEO, ERYTHRO, NR, RIF, STREPTO, CIP, AMP, OF, PB, CEF. CPD, AMX, SZ, PEN, CEF, NA, AMP, CHMP, MET, ERYTHRO, NR, RIF, TET, STREPTO, KAN, NEO. CPD, SZ, AMX, PEN, CHMP, MET, ERYTHRO, CEF, AMP, CIP, PB, RIF, NEO, NR, NA, OF, TET. PEN, CEF, MET, RIF, CIP, PB, AMX, CPD, NR, CHMP, NA, ERYTHRO, KAN, NEO, AMP, OF.	4	16	0.8
17.	CPD, AMX, PEN, AMP, CHMP, MET, ERYTHRO, NR, RIF, SZ, CEF, CIP, KAN, NA, OF, TET, NEO. CPD, AMX, PEN, AMP, CHMP, MET, ERYTHRO, NR, RIF, STREPTO, CIP, KAN, NA, TET, PB, SZ, OF.	2	8	0.85
18.	CPD, PEN, MET, ERYTHRO, NR, RIF, STREPTO, CHMP, CIP, AMX, NA, PB, CEF, NEO, TET, AMP, KAN, GEN.	1	4	0.9
20.	CPD, AMX, PEN, CEF, CHMP, PB, MET, ERYTHRO, RIF, STREPTO, AMP, CIP, GEN, NR, TET, KAN, SZ, NA, NEO, OF.	1	4	1

environments than in control, and the selective pressures shaped the structure of the gene cassette pool, indicating that relative gene transfer potential is higher in the microbial communities of the contaminated environments.

A variety of *bla* genes identified in bacteria derived from different environmental sources such as water or sediments of aquaculture areas (Srinivasan et al., 2005; Jacobs and Chenia, 2007), STPs (Szczepanowski et al., 2004; Leski et al., 2013; Antunes et al., 2006; Taviani et al., 2008), and surface water (Poppe et al., 2006). The environmen-

tal compartments may further serve as reservoirs for β-lactam resistance genes. The *bla* genes are often detected in environmental pathogens including *Aeromonas* (Jacobs and Chenia 2007), *Enterobacter* (Leski et al., 2013), *Salmonella* (Antunes et al., 2006; Moura et al., 2007), *Staphylococcus* (Volkmann et al., 2004), and *Vibrio* spp. (Taviani et al., 2008). AmpC gene encoding β-lactamases has been detected in the microbial isolates from wastewater, surface water and even from drinking water films (Blaak et al., 2010). *bla* genes often coexist with other antimicrobial resistance determinants and can also be associated with mobile genetic elements, increasing the possibility of multidrug resistance and environmental dissemination (Schlüter et al., 2007).

A high antibiotic resistance had been reported in the past two decade (Michael, 2009) and antibiotic resistance still remains a global problem today. High level of antibiotic resistance was observed in this study with twenty antibiotics. From the three sampling sites, 77 isolates of *E. coli* were isolated. All the isolates were tested for their resistance against particular as well as multiple antibiotic resistance. A varied trend of resistance among the isolates was recorded from the three different sampling sites (I, II and III). All isolates showed multiple resistance to antimicrobial agents tested. Of the 100% isolates from sites II and III there was resistance against Amoxicillin, Polymixin B, Methicillin and Ampicillin, Polymixin B, Penicillin, respectively. In the case of site I, 100% resistance was not observed against any antibiotic. Of the >50% isolates were found resistant against most of the antibiotics tested from site I while it was observed against 7 and 6 antibiotics among the isolates from sites II and III, respectively. In the case of multiple resistance, most of the isolates showed multiple antibiotic resistance. All the isolates from all sampling sites showed 11-12 resistance patterns for 20 antibiotics. In the case of site I, 18.5 and 25.6% isolates showed resistance to 11 and 10 antibiotics at a time in 5 and 7 different combinations, respectively, while 12, 16% and 20% isolates from site II showed resistance to 17, 11, and 15 antibiotics at a time in 3, 4 and 5 different combinations, respectively. Of the 4%, isolates from site-III exhibited multiple resistance to 7, 8, 11, 18 and 20 antibiotics at a time in one combination, respectively. However, the high level of *E. coli* resistance to tested antibiotic seems to correspond to the report of Adegunloye (2006). Most of the isolated strains of *E. coli* showed high level of resistance more than other bacteria from the intestinal tract as reported by Esposito and Leone (2007). The bacterial isolates showed high level of antibiotic resistance against all used antibiotics. The result was in agreement with that of Muhammad et al. (2010) who reported that the abuse and misuse of antimicrobial agents for growth promotion and prevention of diseases has impressed a selective pressure that causes discovery of more resistant bacteria.

In another study conducted in Pakistan, the susceptibility pattern of the urinary tract infection causing *E. coli* was studied. The susceptibility pattern of imepenem was 98%, while meropenem was 97%. Gentamicin had a sensitivity of 48%, while Ciprofloxacin was 35% and Cotrimoxazole was 17%. They also concluded that multidrug resistant and ESBL producing *E. coli* was in large proportion in this region (Ullah et al., 2009). The pattern of sensitivity was also affected with the type of infection, as ESBL producers had high rate of resistance to cephalosporin and penicillin groups as compared to non ESBL producers.

CM Jardine (2012) reported that there were larger multiple antibiotic resistance of *E. coli* isolated in urban areas than from rural areas. Ramteke (1997) studied the antibiotic resistance of 448 coliforms isolated from drinking water and their tolerance to heavy metals. More than 90% of metal tolerant isolates showed resistance to one or more antibiotics tested. Parveen et al. (1997) studied total 765 *E. coli* isolates for their multiple-antibiotic resistance profiles with 10 antibiotics and stated antibiotics resistance pattern influenced by geographical condition.

MAR is considered as a good tool for risk assessment. This also gives an idea of the number of bacteria showing antibiotic resistance in the risk zone in the study's routine susceptibility testing. This MAR index also recommended that all isolates, somehow, originated from the environment where antibiotics were over used (Moon, 2013). MAR index values higher than 0.2 were considered to have originated from high-risk sources where antibiotics are often used (Hemen et al., 2012).

In our study we also determined the MAR index of *E. coli* isolates from all three sampling sites. Isolates showed a variation in their MAR index based on sampling sites. Low and high risk MAR were recorded among the *E. coli* isolates from the water samples of the Gomti River. MAR range 0.1-0.7, 0.3-0.9 and 0.35-1.0 were recorded among the isolates from site I (polluted), site II (polluted) and site III (less polluted receiving the treated water near the treatment plant), respectively. No significant difference among the isolates from polluted and less polluted sites was observed regarding their antibiotic (Chitanand et al., 2010)

All *E. coli* strains isolated from river and polluted waters show a high incidence of MAR phenotype. Many investigators have recognized that wastewater treatment plants are the principal recipients of enteric bacteria with multiple antibiotic resistance (Selvaratnum and Kuberger, 2004) and an important site for horizontal gene transfer, by containing nutrients and high concentrations of microorganisms (Sloan et al., 2014).

MAR indexing is likely to provide a useful tool for better risk assessment by identifying contamination from high-risk environments. These investigations suggest that an unexpected increase in the MAR index of *E. coli* isolates from food should prompt an immediate investigation even though the number of *E. coli* organisms present is below the established guideline or standard. The disposal of treated sewage into rivers, lakes or elsewhere may or may

not influence environmental bacterial populations (Sloan et al., 2014). Some studies have found that wastewater treatment can raise or lower the proportions of antibiotic resistant bacteria which carry antibiotic resistance plasmids (Silva et al., 2006). The observation of increased resistance frequency to ampicillin, tetracycline, streptomycin and chloramphenicol after wastewater treatment has previously been reported by Reinthaler et al. (2013).

High-MAR *E. coli* are also the major reservoirs for enteric diseases which are transmitted to human through food and water. It was also found that nitrofurazone-resistant *E. coli* organisms were frequently isolated from the poultry environment but seldom elsewhere. As mentioned earlier, nitrofurozone has very limited use but is allowed in animal feeds for the control of coccidiosis in poultry and bacterial enteritis (scours) in swine. Nitrofurozone may prove to be a useful marker, signalling fecal contamination from this source (Bendall, 2009).

The aim of this study was to establish the microbiological safety of water sources and to provide updated data on resistance index, which may help in identifying the high risk contamination sites in the aquatic environment. The *E. coli* is indicative of general hygienic quality of the water and potential risk of infectious diseases from water.

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REFERENCES

Adegunloye DV (2006). Microorganisms associated with poultry faeces. J. Food Agric. Environ. 4:41-42.

Ahmed W, Goonetilleke A, Gardner T (2010). Implications of faecal indicator bacteria for the microbiological assessment of roof-harvested rainwater quality in southeast Queensland, Australia. Can. J. Microbiol. 56(6): 471-479.

Alpay-Karaoglu S, Ozgumus OB, Sevim E, Kolayli F, Sevim A, Yesilgil P (2007). Investigation of antibiotic resistance profile and TEM-type β-lactamase gene carriage of ampicillin- resistant Escherichia coli strains isolated from drinking water. Annal. Microbiol. 57: 281-288.

Batt AL, Bruce IB, Aga DS (2006). Evaluating the vulnerability of surface waters to antibiotic contamination from varying wastewater treatment plant discharges. Environ. Pollut.142: 295-302.

Bendall JG (2009) Comment on New Reagent for Trace Determination of Protein-Bound Metabolites of Nitrofurans in Shrimp Using Liquid Chromatography with Diode Array Detector. J. Agric. Food Chem. 57(23):11446–11447.

Blaak H, Schets FM, Italiaander R, Schmitt H, de Roda Husman AM (2010) Antibioticaresistente bacteriën in Nederlands oppervlaktewater in veeteeltrijk gebied. RIVM rapport 70371903, 9-10.

Chitanand MP, Kadam TA, Gyananath G, Totewad ND, Balhal DK (2010). Multiple antibiotic resistance indexing of coliforms to identify high risk contamination sites in aquatic environment. Indian J. Microbiol. 50: 216-220.

Dang H, Zhang X, Song L, Chang Y, Yang G (2006). Molecular characterizations of oxytetracycline resistant bacteria and their genes from mariculture waters of China. Mar. Poll. Bull. 52: 1494-1503.

Dierikx C, Essen-Zandbergen AV, Veldman K, Smith H, Mevius D. (2010). Increased detection of extended spectrum beta-lactamase producing *Salmonella enterica* and *Escherichia coli* isolates from poultry. Vet. Microbiol. 145(3-4) 273–278.

Downing T, Imamura H, Decuypere S, Clark TG, Coombs GH, Cotton JA, et al (2011). Whole genome sequencing of multiple*Leishmania donovani* clinical isolates provides insights into population structure and mechanisms of drug resistance. Genome Res. 21: 2143-2156

Drewnowska JM, Swiecicka I (2013). Eco-Genetic Structure of Bacillus cereus sensu lato Populations from Different Environments in Northeastern Poland. PLoS ONE 8(12): e80175. doi:10.1371/journal.pone.0080175.

Esposito S, Leone S (2007). Antimicrobial treatment for Intensive Care Unit (ICU) infections including the role of the infectious diseases specialist. Int. J. Antimicrob. Agents 29: 494-500.

Fischbach MA, Walsh CT (2009) Antibiotics for Emerging Pathogens. Science 325: 1089-1093.

Florea AB (2011). Antimicrobial susceptibility of Eschericia coli isolated from aries river (Romania). Analele Universităţii din Oradea - Fascicula Biologie 18(1): 34-38.

Hemen JT, Johnson JT, Ambo EE, Ekam VS, Odey MO, Fila WA (2012). Multi-Antibiotic Resistance of Some Gram Negative Bacterial Isolates from Poultry Litters of Selected Farms in Benue State. IJST 2(8): 543-547.

Igbinosa EO, Okoh AI (2008). Emerging *Vibrio* species: an unending threat to public health in developing countries. Res. Microbiol. 159(7–8): 495–506.

Jacobs L, Chenia HY (2007) Characterization of integrons and tetracycline resistance determinants in Aeromonas spp. Isolated from South African aquaculture systems. Int. J. Food Microbiol. 114: 295–306.

Jardine CM, Janecko N, Allan M, Boerlin P, Chalmers G, Kozak G, McEwen SA, Reid-Smith RJ (2012) Antimicrobial Resistance in Escherichia coli Isolates from Raccoons (Procyon lotor) in Southern Ontario, Canada. Appl. Environ. Microbiol. 78(11):3873.

Leski TA, Bangura U, Jimmy DH, Ansumana R, Lizewski SE, Li RW, Stenger DA, Taitt CR, Vora GJ (2013) Identification of blaOXA-$_{51}$-like, blaOXA-$_{58}$, blaDIM-$_{1}$, and blaVIM carbapenemase genes in hospital Enterobacteriaceae isolates from Sierra Leone. J. Clin. Microbiol. 51(7): 2435-2438.

Moon H (2013) Efficacy of essential oils as an anti-bacterial agent for the therapeutic management of Clinical MAR Index E. coli. Asiatic J. Biotechnol. Resour. 04 (01) 44-48.

Moura A, Henriques I, Ribeiro R, Correia A (2007). Prevalence and characterization of integrons from bacteria isolated from a slaughter house wastewater treatment plant. J. Antimicrob. Chemother. 60: 1243–1250.

Muhammad M, Muhammad LU, Ambali AG, Mani AU (2010). A survey of early chick mortality on small-scale poultry farms in Jos, Central Nigeria. Int. J. Poult. Sci., 9: 446-449.

Parveen S, Murphree RL, Edmiston L, Kaspar CW, Portier KM, Tamplin ML (1997). Association of multiple antibiotic resistance profiles with point and nonpoint sources of Escherichia coli: in Apalachicola bay. Appl. Environ. Microbiol. 63(7): 2607-2612.

Patoli AA, Patoli BB, Mehraj V (2010) High Prevalence of drug resistant Escherichia coli in drinking water samples from Hyderabad. Gomal. J. Med. Sci. 8 (1):23-26

Poppe C, Martin L, Muckle A, Archambault M, McEwen S, Weir E (2006). Characterization of antimicrobial resistance of Salmonella Newport isolated from animals, the environment, and animal food products in Canada. Can. J. Vet. Res. 70: 105–114.

Ramteke PW (1997). Plasmid mediated cotransfer of antibiotic resistance and heavy metal tolerance in coliforms. Indian J. Microbiol. 37: 177-181.

Riaz S, Faisal M, Hasnain S (2011). Antibiotic susceptibility pattern and multiple antibiotic resistances (MAR) calculation of extended spectrum β- lactamase (ESBL) producing Escherichia coli and Klebsiella species in Pakistan. Afr. J. Biotechnol. 10(33): 6325-6331.

Schlüter A, Szczepanowski R, Pühler A, Top EM (2007). Genomics of IncP-1 antibiotic resistance plasmids isolated from wastewater treatment plants provides evidence for a widely accessible drug resistance gene pool. FEMS Microbiol. Rev. 31: 449–477.

Silva J, Castillo G, Callejas L, López H, Olmos J (2006). Frequency of transferable multiple antibiotic resistance amongst coliform bacteria isolated from a treated sewage effluent in Antofagasta, Chile. Electron. J. Biotechnol. 9(5): 533-540.
Sloan DB, Nakabachi A, Richards S, Qu J, Murali SC, Gibbs RA, Moran NA (2014) Parallel Histories of Horizontal Gene Transfer Facilitated Extreme Reduction of Endosymbiont Genomes in Sap-Feeding Insects. Mol. Biol. Evol. doi:10.1093/molbev/msu004.
Soderstrom A, Osterberg P, Lindqvist A, Jönsson B, Lindberg A, Blide S et al., (2008). A Large Escherichia coli O157 Outbreak in Sweden Associated with Locally Produced Lettuce. Foodborne Pathog. Dis. 5(3): 339-349.
STAS 3001-91 – Water, bacteriological analysis.(Please provide Year)
Taviani E, Ceccarelli D, Lazaro N, Bani S, Cappuccinelli P, Colwell RR, Colombo MM (2008). Environmental Vibrio spp., isolated in Mozambique, contain a polymorphic group of integrative conjugative elements and class 1 integrons. FEMS Microbiol. Ecol. 64: 45–54
Tsiodras S, Kelesidis T, Kelesidis I, Bauchinger U, Falagas ME (2008). Human infections associated with wild birds. J. Infect. 56: 83-98.
Ullah F, Malik SA, Ahmed J (2009). Antibiotic susceptibility pattern and ESBL prevalence in nosocomial Escherichia coli from urinary tract infections in Pakistan. Afr. J. Biotechnol. 8: 3921-3926.
Vulliet E, Cren-Olive C (2011) Screening of pharmaceuticals and hormones at the regional scale, in surface and groundwaters intended to human consumption. Environ. Pol. 159 (10):2929–2934.
Wright MS, Baker-Austin C, Lindell AH, Stepanauskas R, Stikes HW, McArthur JV (2008). Influence of industrial contamination on mobile genetic elements: class 1 integron abundance and gene cassette structure in aquatic bacterial communities. ISME J. 2: 417–428
Wright MS, Peltier GL, Stepanauskas R, McArthur JV (2006). Bacterial tolerances to metals and antibiotics in metal-contaminated and reference streams. FEMS Microbiol. Ecol. 58: 293–302.

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Isolation and characterization of endophytic plant growth-promoting bacteria (PGPB) associated to the sodicity tolerant polyembryonic mango (*Mangifera indica* L.) root stock and growth vigour in rice under saline sodic environment

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In the recent times, there has been a reversed interest in the search of plant growth promoting endophytes (PGPE) for sustainable crop production. Mango is an economically important fruit crop, which is highly sensitive to saline environment. In the present study the importance of PGPR in growth promotion and their ability to elicit 'induced systemic tolerance' against abiotic (sodicity) stresses has been documented. Sixteen (16) putative of endophytic bacteria were isolated from sodicity tolerant polyembryonic mango root stock of GPL-3 and ML-4 from Shivari experimental farm, Central Soil Salinity Research Institute, Regional Research station, Lucknow, Uttar Pradesh, India. These endophytic bacteria were characterized using morphological and biochemical parameters and assessed for their plant growth promoting rhizobacteria (PGPR) traits like indole acetic acid (IAA) production, hydrogen cyanide (HCN) production, phosphate solubilization and siderophore production with 2.5 M NaCl concentration. The results based on specific biochemical characters revealed that the endophytic bacteria belonged to 14 different species and two uncultured organisms comprising five genera. Based on multifunctional properties under saline environment, four isolates were further selected to determine the PGP as vigor index of rice seedlings in pot culture experiments under saline sodic soils of pH 9.35 and EC 4.2. Of the bacteria tested, the isolate CSR-M-16 showed increased root and shoot length of rice followed by CSR-M-8, CSR-M-9 and CSR-M-6. The outcome of this research proves plausible practical applicability of these PGPB for crop production in saline sodic environment.

Key words: Endophytes, salt tolerant, PGP bacteria, mango root stock, polyembryony.

INTRODUCTION

Plant tissues are not sterile-spaces; within them there are different species of bacteria known as endophytes. Most of these microorganisms are not pathogenic to the host plant. Moreover, the association between the plant and

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endophytic bacteria is very often mutualistic. Endophytes enter plant tissue primarily through the root zone; however, aerial portions of the plants such as flowers, stems and cotyledons may also be used for entry (Kobayashi and Palumbo, 2000). Once they get inside the plant tissue, endophytic bacteria may either remain localised at the point of entry or spread throughout the plant (Hallmann et al., 1997). Selections of microbial isolates from naturally stressed environment or rhizosphere are considered as possible measures for improving crop health which can control diseases and also promote plant growth (Lugtenberg and Kamilova, 2004; Mayak et al., 2004).

Worldwide, salinity is one of the most severe abiotic stresses that limit crop growth and productivity; about 20% of world's irrigated land is salt affected, with 2,500-5,000 km^2 of production lost every year as a result of salinity (Nellemann, 2009). As reported, (Gupta and Abrol, 2000) 60% of salt affected soils are of sodic and saline sodic in nature which has increased steadily over decades in the northwest plains of the Indo-Gangetic basin and in China's Yellow River basin. High alkalinity (pH > 8.5) and high exchangeable sodium percentage (ESP) of the soil render it inhospitable for normal crop production and there is minimal bioproductivity in such soil (Chhabra, 1995). The utilization of salt-affected soil for agriculture has become necessary to meet the rise in food demand and one possible strategy to counteract the adverse effect of salinity is to exploit the avenues of bio-agents or bio-inoculants (Egamberdieva, 2012). Under salt stress, PGPR have shown positive effects in plants on parameters such as germination rate, tolerance to drought, weight of shoots and roots, yield and plant growth (Raju et al., 1999). The combination of IAA production ability (Goldstein et al., 1999), phosphorous solubilisation (Gyaneshwar et al., 1998) and siderophore production (Duffy and Défago, 1999) of bacteria aid the plant rhizosphere in enhancing the nutrient absorption potential even under sodic environment for enabling economic production of commercial horticultural crops (Damodaran et al., 2011). This has been extensively attracting attention due to their efficacy as biological control and growth promoting ability in many crops. Though the researchers earlier have worked on isolation of salt (NaCl) tolerant rhizobacteria from halophilic environment where the conductivity (EC) of the soils is > 4 dS m^{-1}. Little is known about their tolerance to saline sodic environment where the soils are severely affected by high pH characterized by high Na^+ in the soil solution phase as well as on cation exchange complex (Qadir and Schubert, 2002), exhibiting unique structural problems.

Mango is more sensitive to salinity and sodicity particularly at early growth stage which necessitates the requirement of salt tolerant true to type polyembryony rootstocks. According to Kadman et al. (1976) and Gazit and Kadman (1980, 1983), mango rootstock 13-1 very popular in Israel, is tolerant to low quality waters.

However, in the Southeast of the Spanish peninsula and in the Canary Islands this is not popular and the most commonly used rootstocks are Gomera-1 and Gomera-3 (Galan and Garcia, 1979; Galan and Fernandez, 1988). Mango is considered sensitive to saline conditions (Maas, 1986), leading to scorched leaf tips and margins, leaf curling, and in severe cases reduced growth, abscission of leaves, and death of trees (Jindal et al., 1976). The information concerning the salt tolerance of mango rootstocks is lacking, particularly on the impact of salinity on fruit yield (Ayers and Westcot, 1989; Maas and Grattan, 1999). Further, it was suggested that polyembryony rootstocks are more preferred for mango breeding studies for abiotic stress (Mohammad et al., 2001; Varu and Barad, 2010). The nucellar seedlings of the polyembryony varieties produce true to type parent character and is also known for their performance under stress environment (Damodaran et al., 2011). Consequently, sustaining and enhancing the growth under salt affected field of polyembryonic mango plants have become a major focus of research. The growth and performance of ployembryonic mango plants in the field are adversely affected by sodium toxicity. Therefore the present study is focused to survey plants habitat of the sodic lands, isolate the endophytic bacteria from growing polyembryonic mango root stock under saline sodic environment, characterize them by morphological and biochemical means and screen them for their PGP under salinity and sodicity environment.

MATERIALS AND METHODS

Plant material

For the isolation of endophytes, salt tolerant root stock of polyembryonic mango (*Mangifera indica*) accessions ML-2 and GPL-3 grown in sodic soil were collected from Shivari experimental farm (22°41S 47°33W) of Central Soil Salinity Research Institute (ICAR), Regional Research Station, located in Lucknow, Uttar Pradesh, India.

Physico-chemical analysis of soil

The collected sodic soil sample (pot culture experiment) was analyzed for physicochemical parameters like pH and conductivity. The pH and EC of the soil extract was determined potentiometrically by an ORION ion analyzer (5 star series) using a pH electrode and conductivity electrode.

Isolation of endophytic bacteria

The uprooted healthy sodicity tolerant mango plants were briefly washed with sterile water and the root, stem and leaves were cut into 2-3 cm long pieces. These pieces were rinsed in sterile water and then surface disinfected by soaking in 70% ethanol for 30 s and then treated with sodium hypochlorite (3-5% available chlorine) for 3 min. Samples were exhaustively rinsed with sterile water so that all the epiphytic microorganisms could be removed. Next, they were cut longitudinally with a sterile scalpel and laid, with the exposed

inner surface facing downwards, on plates of sterile nutrient agar (NA) as adapted by Hung and Annapurna (2004). As a control, uncut, surface-disinfected stem pieces and non-disinfected pieces were also placed on the same agar. All plates were incubated for 48 h at 27°C.

Bacterial identification

The isolates were initially categorized into two broad groups based on Gram staining by Hucker's modified method (Rangaswami and Bagyaraj, 1993). Morphological and cultural characters of the isolates were used for further grouping. Based on the results of various biochemical tests such as indole production, nitrate reduction, citrate-utilization, hydrolysis of lactose, cellobiose and sucrose, the catalase activity of the organisms were performed to identify on generic level by using specific biochemical tests (Cappunccino and Sherman, 1992).

***In vitro* analysis for the identification of PGP activity bacteria**

Screening of NaCl tolerance

Nutrient broth (10 mL) was supplemented with NaCl so as to give 0.1-3.5 M NaCl. All the isolates were grown in tubes and incubated on rotary shaker (150 rpm) at 37°C. Bacterial growth was determined as OD_{600} to find out NaCl tolerance. Actively growing bacteria were then serially adapted to 2.0 M NaCl concentration.

Phosphate solubilization by the selected salt tolerant isolates

NaCl tolerant isolates were checked for phosphate solubilization by the ability to solubilize inorganic phosphate. For this, Pikovskaya's agar amended with 2.0 M NaCl and calcium phosphate was used. Spot inoculation of the tolerant isolates was done in the center of the medium and kept for incubation at 28°C for 48-72 h. The appearance of transparent halo zone around the bacterial colony indicated the phosphate solubilizing activity of the bacteria.

IAA production by the selected tolerant isolates

Indole acetic acid (IAA) production was detected as described by Brick et al. (1991). Bacterial cultures were grown for 72 h in nutrient broth medium containing supplement of 2.0 M NaCl at 36- 38°C. Fully grown cultures were centrifuged at 3,000 rpm for 30 min. The supernatant (2 mL) was mixed with 2 drops of orthophosphoric acid and 4 mL of Salkowski reagent (50 mL, 35% of perchloric acid and 1 mL 0.5 M $FeCl_3$ solution). Development of pink colour indicated IAA production. The amount of IAA produced by rhizobacteria was estimated quantitatively by Salkowski method (Dubey and Maheshwari, 2012). The cultures were incubated in peptone broth together with tryptophan for 24 and 48 h, and IAA production was estimated.

HCN production by the selected tolerant isolates

Production of HCN was detected according to the method of Lorck (1948). Briefly, nutrient broth was amended with 4.4 g glycine L^{-1} and 2.0 M NaCl. Bacteria were streaked on modified agar plate. A Whatman filter paper no. 1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed at the top of the plate. Plates were sealed with parafilm and incubated at 36±2°C for four days. Development of orange to red colour indicated HCN production.

Siderophore production by the selected tolerant isolates

Bacterial culture (48 h) was streaked on nutrient agar amended with an indicator dye and 2.0 M NaCl. The tertiary complex chrome-azurol-S (CAS) /Fe^{3+} / hexadecyl trimethyl ammonium bromide served as an indicator. Change of blue color of the medium surrounding the bacterial growth to fluorescent yellow indicated production of siderophore. The reaction of each bacterial strain was scored either positive or negative to the assay (Schwyn and Neilands, 1987).

Sodium uptake by the tolerant isolates

The screened isolates for salt tolerance were further studied for sodium uptake pattern. To determine the sodium uptake pattern, the isolates were grown overnight at 37°C in Nutrient broth containing different NaCl concentration (0.1, 0.5, 1.0, 1.5, 2.0 and 2.5 M). After 24 h of incubation the bacterial cells were harvested by centrifugation and bacterial pellet obtained was washed with sterile distilled water to remove the traces of medium. Washed pellet was digested overnight with 0.1N HCl at room temperature. Samples were centrifuged and supernatant was taken for the estimation of sodium uptake by bacterial cells using flame photometer.

***In vivo* plant growth promotion under saline sodic soil conditions**

The endophytic bacteria were grown on nutrient broth with constant shaking on rotary shaker at 150 rpm for 48 h at room temperature (28±2°C) and were harvested by centrifugation at 6,000 rpm for 15 min. The bacterial cells were resuspended in PB (0.01M, pH 7.0). The concentration was adjusted to approximately 10^8 CFU (OD_{595}=0.3) and used as inoculums for treating rice seeds (Thompson, 1996). Plant growth promoting activities of bacterial strains were assessed based on the seedling vigor index of rice seed under pot culture studies in soil of pH 9.35 ECe (4.2), Na^+ (23.50 meq L^{-1}) and sodium adsorption ratio (SAR) 19.36. Sodium (Na^+) was determined by flame photometer (Richards, 1954) while SAR was determined by following the generic equation:

$$SAR = \frac{Na}{\sqrt{(Ca + Mg)/2}}$$

The vigor index was calculated by using the formula as described by Abdul Baki and Anderson (1973).

Statistical analysis

The pot culture experiment on assessing vigor index in rice seeds treated with bacterial isolates was conducted in completely randomized design (CRD) with three replications and the data was analyzed using SAS 9.2 version. Prior to analysis of variance the percentage values of germination were arcsine transformed.

RESULTS

Endophytic bacteria were isolated from the root, stem and leaves of the sodicity tolerant polyembryonic mango accessions (GPL-3 and ML-4) grown in sodic soils of pH 9.51. The population of the endophytic bacteria in the

Table 1. Endophytic bacterial population isolated from sodicity tolerant polyembryonic mango accessions GPL-3 and ML-4 (CFU g^{-1} FW).

Place of collection	Accession	GPS data	Plant Part	Bacterial population (CFU g^{-1} FW)	Number of phenotypes selected
Guptapara	GPL-3	N 11°, 32', 15.6 E 92°, 39', 0.7	Stem	4.4×10^{-4}	2
			Leaves	4.8×10^{-4}	1
			Root	9.0×10^{-4}	4
Manjeri	ML-4	N 11°, 32', 32',7.6 E 92°, 39', 5.6	Stem	5.8×10^{-4}	2
			Leaves	5.5×10^{-4}	1
			Root	10.5×10^{-4}	6
Total no of isolates					16

Table 2. Phenotypic characteristic as determined by Gram staining of selected bacterial isolates obtained from both salt tolerant (sodicity) tolerant polyembryonic mango accessions GPL3 and ML-4.

Salt tolerant polyembryonic mango accessions	Plant parts	No of Gram-positive isolates	No of Gram-negative isolates	Group of isolates	Total gram-positive and gram-negative isolates	Lowest denomination ratio of Gram-positive and gram-negative	Rod:cocci:spirillum:Oval
GPL-3	Stem	2	0	CSR-M-05, CSR-M-12	7	2.2:1	10:1:2:3
	Leaves	0	1	CSR-M-10			
	Root	4	0	CSR-M-01, CSR-M-02, CSR-M-08, CSR-M-09			
ML-4	Stem	1	1	CSR-M-03, CSR-M-11,	9		
	Leaves	1	0	CSR-M-13			
	Root	3	3	CSR-M-04, CSR-M-06, CSR-M-07, CSR-M-14, CSR-M-15, CSR-M-16			
Total Isolates		11	5				

Various plant tissues of the sodicity tolerant polyembryonic mango root stock is shown in Table 1. In both accessions, bacterial growth was observed. The population of bacteria in root ranged from 9.0 to 10.5×10^4 cfu followed by stem which was 4.4 to 5.8×10^4 cfu and 4.8 to 5.5×10^4 cfu in leaves (Table 1). About 40 bacterial isolates were collected, among them 16 were selected based on their ability to survive at 2.5 M NaCl concentration for further experiment. Further they were screened for Gram nature, pigmentation, colony colour and morphological characteristic (Tables 2 and 3). Among 16 isolates, CSR-M-03, CSR-M-05, CSR-M-06, CSR-M-08, CSR-M-14 and CSR-M-15 were yellowish to creamy yellow colour while the others were white to creamy white. Pigmentation was absent in most of the isolates except CSR-M-03, CSR-M-04, CSR-M-14 and CSR-M-15 which displayed yellow to greenish yellow pigments production. The isolates were of lowest denomination ratio of Gram-positive (68.75 %) and Gram negative (31.25 %) with a range of 2.2:1 ratio under microscope observation. The shapes ranged with a ratio of 10:1:2:3 for rod: cocci:spirillum:oval where rods were found to be the major phenotypes (Table 2). Carbohydrate utilization indicated that 2 out of 16 isolates were

Table 3. Morphological characteristics of bacterial endophytes.

Experiment Procedure	Observations and result															
	M-1	M-2	M-3	M-4	M-5	M-6	M-7	M-8	M-9	M-10	M-11	M-12	M-13	M-14	M-15	M-16
Colour	W	W	Y	CW	Y	Y	W	Y	PW	W	W	W	W	CY	Y	W
Shape	R	R	R	R	R	O	R	C	S	S	O	R	R	O	R	R
Elevation	Cv	Ra	Cv	Ra	Ra	Ra	Ra	Ra	F	Ra	Ra	Ra	Ra	Ra	Ra	Ra
Pigmentation	NP	NP	Y	G	N	N	N	N	N	N	N	N	N	Y	Y	N

W=White; Y= yellow; CW= creamy white; PW= Powdery white; CY= Creamy yellow; S= spherical; C= cocci; R= rod; Cv= convex; Ra= raised; F= flat; Y= yellow; NP= no pigmentation.

able to produce acid and hydrolyses glucose, lactose, sucrose and fructose while 12 isolates were able to assimilate nitrate and citrate utilization and produced catalase enzyme and 7 were positive for indole production (Table 4).

Salt tolerance traits

On screening the 16 bacterial isolates for growth in different NaCl concentrations; four isolates growing luxuriantly in 2.5 M NaCl concentration were selected for further evaluations. These four isolates were further analysed for their sodium uptake pattern (Figure 1) at different molar (M) concentration of NaCl. The four isolates had an increasing sodium (Na^+) uptake up to 2.5 M NaCl compared to other isolates. However, four isolates CSR-M-06, CSR-M-08, CSR-M-09 and CSR-M-16 identified as *Bacillus pumilus* and *Bacillus subtilis* showed higher uptake of Na^+ (23,400 ppm g^{-1}, 19,240 ppm g^{-1}, 19,440 ppm g^{-1} and 19,540 ppm g^{-1} of fresh weight respectively) at 2.0 M NaCl concentration.

PGP traits with supplement of NaCl

In PGP traits (Table 5) with supplement of 2.0 M sodium chloride concentration, four isolates showed IAA, siderphore production and phosphate solubilization and none of the isolate showed HCN production. The isolates CSR-M-06 (49.5 µg mL^{-1}), CSR-M-09 (25.2 µg mL^{-1}) and CSR-M-16 (74.0 µg mL^{-1}) had extensive formation for IAA production. Further, this four isolates CSR-M-06, CSR-M-08, CSR-M-09 and CSR-M-16 showed higher phosphate solubilisation. The results are reported as ranking in Table 5.

Plant growth promotion potential of salt tolerant bacteria under in vivo condition

The four isolates were furthermore screened for plant growth potential (PGP) in saline-sodic soils of pH 9.35 and EC 4.2 dS m^{-1} under pot culture experiment with rice seed. All four isolates (CSR-M-06, CSR-M-08, CSR-M-09 and CSR-M-16) showed plant growth enhancing activities with the germination percentage of 88.33, 91.66, 86.66 and 93.33 %. Significantly higher shoot and root growth was observed in CSR-M-16 followed by CSR-M-8, CSR-M-6 and CSR-M-9 with higher vigour index (4,675.8, 4,393.5, 4,260.4 and 3,992.1, respectively). The efficiency of isolates is shown as ranking in Table 6.

DISCUSSION

A detailed screening of polyembryonic mango accessions screened in sodic soils, the sodicity tolerant polyembryonic mango root stock of ML-4 and GPL-3 isolate and identification of the endophytic bacteria that could express plant growth promotion (PGP) traits at high salt concentrations were done. The study indicates that there was reduction in bacterial diversity with increase in soil pH. This agrees with the finding of earlier researcher (Borneman et al., 1996) which also stated the reduction of bacterial diversity under environmental stress such as salinity. In our study, we isolated 16 isolates from sodicity tolerant polyembryonic mango root stock. Majority of the bacterial isolates were identified as *Bacillus* spp. based on morphological and biochemical observations. Earlier studies (Tank and Saraf, 2010) also indicated the dominant of genera such as *Bacillus sp* and *Pseudomonas sp* in saline soils PGP activity of the bacteria present in the rhizosphere is found to exert beneficial effects on plant growth mechanism. Several mechanisms such as production of phytohormones, supperssion of deleterious organisms, production of IAA, activation of phosphate solubilization and promotion of the mineral nutrient uptake are believed to be involved in plant growth promotion by PGPR (Glick et al., 1995). Auxin is the most effective plant growth hormone and among them IAA is a common one. IAA may function as important signal molecule in the regulation of plant development (Usha Rani et al., 2012).

Table 4. Identification of endophytic bacterial isolates based on bio-chemical tests.

Parameter	M-1	M-2	M-3	M-4	M-5	M-6	M-7	M-8	M-9	M-10	M-11	M-12	M-13	M-14	M-15	M-16
Gram staining	-ve	-ve	-ve	+ve	+ve	+ve	-ve	+ve	-ve	-ve	-ve	+ve	+ve	+ve	-ve	+ve
Indole production	+	-	+	-	-	-	+	+	-	-	-	-	+	+	+	-
Catalyse	+	+	-	+	+	+	+	+	-	-	+	-	-	+	+	+
Urease	+	-	-	-	-	-	-	-	-	+	+	-	-	-	+	-
Nitrate	+	-	+	-	+	+	+	+	-	-	-	-	-	-	-	+
H_2S production	-	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-
Citrate utilization	-	+	+	-	+	-	-	-	-	-	-	+	-	+	-	+
Arabinose	-	-	-	+	-	+	+	-	+	+	-	+	+	-	+	+
Trehalose	-	-	-	+	-	+	-	-	+	+	+	+	+	+	+	+
VP	-	-	-	-	+	+	-	-	-	-	-	+	+	-	-	+
ONPG	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Mannitol	+	-	-	-	+	+	-	+	-	-	+	-	-	+	+	+
Malonate	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-
Glucose	A^-	A^+	A^-	A^-	A^+	A^+	A^+	A^-	A^+	A^-	A^+	A^-	A^+	A^-	A^-	A^-
Lactose	A^+	A^-	A^+	A^-	A^+	A^-	A^-	A^-	A^-	A^-	A^+	A^-	A^-	A^+	A^+	A^-
Sucrose	A^-	A^-	A^-	A^+	A^+	A^-	A^-	A^+	A^-	A^-	A^+	A^+	A^-	A^-	A^-	A^+
Fructose	A^-	A^-	A^-	A^-	A^-	A^-	A^-	A^-	A^-	A^-	A^+	A^-	A^-	A^-	A^-	A^-
Cellobiose	A^-	A^-	A^-	A^-	A^-	A^-	A^-	A^-	A^-	A^-	A^-	A^-	A^-	A^-	A^-	A^-
Raffinose	A^-	A^-	A^-	A^+	A^-	A^-	A^-	A^-	A^-	A^-	A^-	A^-	A^-	A^-	A^-	A^-
Identification of the isolates	A	B	C	D	E	F	G	H	I	J	K	M	L	N	O	P

A = *Agrbacterium tumifaciensis;* B = *Oceanobacillus sp;* C = *Psuedomonas aeroginosa;* D = *Ocenobacillus caeni;* E = *Bacillus sp;* F = *Bacillus coagulans* G = *Brevibacillus borstelensis;* H = *Bacillus megatarium;* I = *Bacillus pumilus;* J = *Unculture organism;* K = *Bacillus amloliquefaciens subsp;* L = *Unculture organism*; M = *Brevibacillus thermoruber*; N = *Bacillus lehensis*; O = *Agrobacterium fabrum;* P = *B. subtilis*; A^+ = Acid producers; A^- = No acid producers.

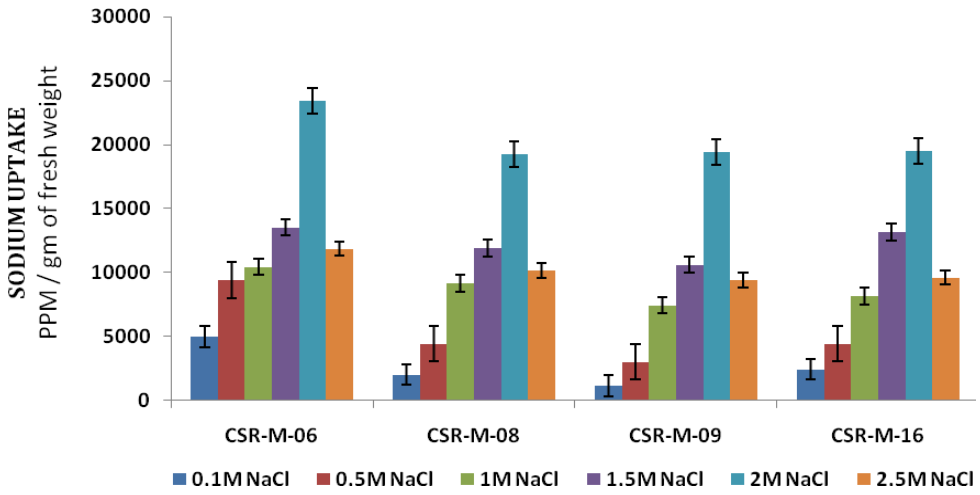

Figure 1. Sodium uptake pattern of the elite PGP isolates of sodicity tolerant polyembryonic mango root stock (vertical bars indicate ± standard error).

Among the isolates under study, only four isolates (CSR-M-6, CSR-M-8, CSR-M-9 and CSR-M-16) exhibited IAA production. Phosphorous (P) is an essential nutrient for plant growth, development and is typically insoluble or poorly soluble in soils under salt stressed conditions (Harrison et al., 2002). Some of the bacteria are known to

Table 5. Assessment of plant growth promoting properties of endophytic bacteria from salt tolerant polyembryonic mango accessions (GPL-3 and ML-4).

Bacterial Isolate	Plant growth promoting properties			
	IAA production ($\mu g\ mL^{-1}$)	Siderophore production	HCN production	PO_4 Solubilisation
CSR-M-6	49.5	+++	+	+++
CSR-M-8	65.7	+++	+	+++
CSR-M-9	25.2	++	+	-
CSR-M-16	74.0	+++	+	+++

IAA, Indole-3-acidic acid; HCN, hydrogen cyanide; PO_4, phosphate; -, No production; +, 0.3-0.5 cm; ++, 0.6-0.9 cm; +++, >1 cm

Table 6. Plant promotion activity of rice seedling vigour and inoculation endophytic bacteria treated rice seed under sodic soil.

Bacterial isolate	Root length (cm)	Shoot length (cm)	Root dry weight (g)	Shoot dry weight (g)	% Germination	Vigour index
CSR-M-6	12.8^{b}	35.5^{b}	0.44^{a}	1.68^{b}	88.33^{b}	4260.45^{b}
CSR-M-8	12.3^{b}	35.6^{b}	0.34^{c}	1.67^{b}	91.66^{a}	4393.57^{b}
CSR-M-9	12.1^{b}	34.0^{c}	0.38^{b}	1.86^{a}	86.66^{b}	3992.14^{c}
CSR-M-16	13.6^{a}	36.5^{a}	0.38^{b}	1.75^{b}	93.33^{a}	4675.83^{a}
Control	3.7^{c}	8.31^{d}	0.14^{d}	0.12^{c}	6.67^{b}	80.22^{d}

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

improve the solubilization of the fixed soil phosphorous and applied phosphates, resulting in higher yields even under stress conditions (Banerjee et al., 2010). The four endophytic bacterial isolates showed *in-vitro* phosphate solubilization activity. Ability to solubilize various insoluble phosphates is always desirable attribute for a competent PGPR. Phosphate solubilization by *Bacillus* sp. isolated from salt stressed environment had been observed by earlier researchers (Son et al., 2006). Siderophore chelates iron and other metals thereby contributes in disease suppression and acquisition of Fe^{2+} to plants for increasing the crop growth under stressed conditions (Hofte et al., 1992; Duffy and Défago, 1999). The side-rophore production ability was found to be a productive PGPR trait for selection. Hydrogen cyanide (HCN) is an important attribute of PGPR which influences plant growth indirectly and strengthen the host disease resistance mechanism (Schippers et al., 1990). In the present study, four isolates were found to produce HCN. Majority of the PGPR properties producing bacteria were identified to be of genus *Bacillus* sp.

These four isolates were found to be tolerant to high salt concentration (2.5 M NaCl) and also showed higher uptake of sodium when cultured under *in-vitro* conditions at this concentration. It has also been reported previously that bacteria isolated from saline soil are more likely to withstand salinity conditions (Upadhyay et al., 2009). On the other hand, if such bacteria also possess plant growth promoting traits they would be ideal for use in sustainable agriculture (Egamberdiyeva and Islam, 2008). Based on biochemical parameters, the four bacterial isolates CSR-M-6, CSR-M-8, CSR-M-9 and CSR-M-16 identified as *Bacillus coagulans, Bacillus megatarium, Bacillus pumilus* and *Bacillus subtilis* respectively exhibited all the *in-vitro* PGPR characteristics like IAA, HCN, siderophore and phosphate solubilization production. Production of IAA, siderophore, phosphate solubilization had been observed in *Bacillus sp*. in earlier studies (Xie et al., 1996; Loper and Henkels, 1997). Furthermore, in the current experiment, the vigor index studies in rice seeds treated with salt tolerant isolates showed that the isolates CSR-M-6, CSR-M-8, CSR-M-9 and CSR-M-16 are potential growth promoter even under saline-sodic conditions apart from salinity. Though PGPR are more commonly known to induce resistance against pathogen infection, reports are now available on their ability to elicit 'induced systemic tolerance' against abiotic stresses (Usha et al., 2011).

Results of our study suggest that sodicity tolerant polyembryonic mango root stock is naturally associated with a variety of endophytic bacteria, which have NaCl tolerance capacity and exhibited PGPR traits like IAA production, phosphate solubilizaiton. The earlier finding (Cohen et al., 2008; Glick et al., 1998) suggested a strong relationship between rhizobacterial capabilities and colonized plant tolerance to salt stress. Therefore, we could speculate about an "endophytic consensus" generated between four strains that colonize the plant in which everyone expresses one or more mechanisms to jointly determine a global response to promote plant

growth or regulate under extreme saline conditions.

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REFERENCES

Abdul Baki AA, Anderson JD (1973).Vigour determination in soybean seed by multiple criteria. Crop Science 13:630-633.

Ayers RS, Westcot DW (1989). Water quality for agriculture, Revised edition 1, Food and Agriculture Organization of the United Nations (FAO).

Banerjee G, Scott-Craig JS, Walton JD (2010). Improving enzymes for biomass conversion: a basic research perspective. Bioenerg. Res. 3:82-92.

Borneman J, Skroch PW , O'Sullivan KM , Paulus JA , Rumjanek NG, Jansen JL, Nienhuis J, Triplett EW (1996). Molecular microbial diversity of an agricultural soil in Wisconsin. Appl. Environ. Microbiol. 62:1935-1943.

Brick JM, Bostock RM, Silversone SE (1991). Rapid in situ assay for indole acetic acid production by bacteria immobilized on nitrocellulose membrane. Appl. Environ. Microbiol. 57:535-538.

Cappunccino JC, Sherman N (1992). In: Microbiology: A Laboratory Manual 3rd, New York: Benjamin/Cumming Pub. Co.

Chhabra R (1995). Nutrient requirement for sodic soil. Fertil. News 40:13-21.

Cohen A, Bottini R, Piccoli P (2008). *Azospirillum brasilense* Sp 245 produces ABA in chemically defined culture medium and increases ABA content in *Arabidopsis* plants. Plant Growth Regul. 54:97-103.

Damodaran T, Rai RB, Mishra VK, Sharma DK, Ram RA, Rai S, Kumar H (2011). Integrated farming System and livelihood security-An approach. CSSRI, Karnal pp. 1-108.

Dubey RC, Maheshwari DK (2012). Practical Microbiology. 2nd ed., S. Chand & Co. P Ltd, New Delhi, p. 413. ISBN: 81: 219-2559-2.

Duffy BK (1999). Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains. Appl. Environ. Microbiol. pp. 2429-438.

Egamberdieva D (2012). *Pseudomonas chlororaphis*: a salt-tolerant bacterial inoculant for plant growth stimulation under saline soil conditions. Acta Physiol. Plantarum. 34:71-56.

Egamberdiyeva D, Islam KR (2008). Salt-tolerant rhizobacteria: plant growth promoting traits and physiological characterization within ecologically stressed environments. In: Ahmad I, Pichtel J, Hayat S (eds) Plant-bacteria interactions- strategies and techniques to promote plant growth. wiley, Weilheim. 257-281.

Galan SV, Garcia SJ (1979). Pasado, presente future del mango en Canarias. I.N.I.A., M.A.P.A.. p. 38.

Galan SV, Fernandez GD (1988). El cultivo del mango en Canarias, I parte. Fruticultura Profesional 16:13-16.Gazit S, Kadman A (1983). Selection of mango rootstocks for calcareous soils and saline irrigation water in the Arava. In: Institute of Horticulture. Volcani Center. Scientific activities, Special publication 222:93.

Gazit S, Kadman A (1980). Mango rootstock selection. Hort. Sci. 15(5):69.

Glick B, Penrose D, Li J (1998) A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. J. Theor. Biol. 190:63-68.

Glick BR (1995). The enhancement of plant growth by free living bacteria. Can. J. Microbiol. 41:109-114.

Goldstein AH (1995). Evidence for mutualism between a plant growing in a phosphate- limied desert environment and a mineral phosphate solubilizing rhizobacteria. pp. 295-300.

Gupta RK, Abrol IP (2000). Salinity build-up and changes in the rice-wheat system of the Indo-Gangetic Plains. Exp. Agric. 36:273-284.

Gyaneshwar P, Naresh KG, Parekh LJ (1998). Role of soil microorganisms in improving P nutrition of plants. Plant Soil. 245:83-93.

Hallmann J, Hallmann QA, Mahaffee WF, Kloepper JW (1997). Bacterial endophytes in agricultural crops. Can. J. Microbiol. 43:895-914.

Harrison MJ, Dewbre GR, Liu J (2002). A phosphate transporter from *Medicago truncatula* involved in the acquisition of phosphate released by arbuscular mycorrhizal fungi. Plant Cell 14:2413-2429.

Hofte M, Boelens J, Verstraete W (1992). Survival and root colonization of mutants of plant growth promoting *Pseudomonads* affected in siderophore biosynthesis or regulation of siderophore production. J. Plant Nutr. 15:2253-2262.

Hung PQ, Annapurna K (2004). Isolation and characterization of endophytic bacteria in soybean (*Glycine* sp.). Omon Rice12:92-101.

Jindal PC, Singh JP, Gupta OP (1976). Studies on salt tolerance in mango injurious effect of salts on young mango seedlings. Progr Hortic. 8:65-74.

Kadman A, Gazit S, Ziv G (1976). Selection of mango rootstock for adverse water and soil conditions in arid areas. Acta Hort. 57:81-88.

Kobayashi DY, Palumbo JD (2000). Bacterial endophytes and their effects on plants and uses in agriculture. In: Microbial endophytes. (Bacon CW, White JF, eds). Marcel Dekker Inc., New York. pp. 199-233.

Loper JE, Henkels MD (1997). Availability of iron to *Pseudomonas fluorescence* in rhizosphere and bulk soil evaluated with an ice nucleation reporter gene. Appl. Environ. Microbiol. 63:99-105.

Lugtenberg BJJ, Kamilova FD (2004). Rhizosphere management: microbial manipulation for biocontrol. In Goodman RM (eds) Encyclopedia of plant and crop science. Marcel Dekker, New York. pp. 1098-1101.

Maas EV (1986). Salt tolerance of plants. In: Applied Agricultural Research, Springer-Verlag, NY, USA. 1:12-26.

Maas EV, Grattan SR (1999). Crop yields as affected by salinity. In R. W. Skaggs and J. van Schilfgaarde (eds) Agricultural Drainage. Agron. Monograph. ASA, CSSA, SSA, Madison, WI 38:55-108.

Mayak S, Tirosh T, Glick BR (2004). Plant growth-promoting bacteria that confer resistance in tomato and pepper plants to salt stress. Plant Physiol. Biochem. 167:650-656.

Muhammad U, Fathima B, Muhammad J (2001). Breeding in mango. Int. J. Agric. Biol. 3:522-526.

Qadir M, Schubert S (2002). Degradation processes and nutrient constraints in sodic soils. Land Degr. Dev. 13:275-294.

Raju NS, Niranjan SR, Janardhan GR, Prakash HS, Mathur SB Agents. pp. 206-212.

Rangaswami G, Bagayaraj DJ (1993). Microbial biotechnology. In: Agricultural Microbiology. Prentice Hall of India Pvt. Ltd., New Delhi. 389-405.

Richards LA (Ed.) (1954). Diagnosis and improvement of saline and alkali soils. US Salinity Lab, US Department of Agriculture Handbook 60, California, USA.

Schippers B, Bakker AW, Bakker R, Van Peer R (1990). Beneficial and deleterious effects of HCN-producing *pseudomonads* on rhizosphere interactions. Plant Soil 129:75-83.

Schwyn B, Neilands JB (1987). Universal chemical assay for detection and determination of siderophore. Anal. Biochem. 160:47-56.

Son HJ, Park GT, Cha MS, Heo MS (2006). Solubilization of insoluble inorganic phosphates by a novel salt- and pH tolerant *Pantoea agglomerans R-42* isolated from soybean rhizosphere. Biores. Technol. 97:204-210.

Tank N, Saraf M (2010). Salinity resistant plant growth promoting rhiz obacteria ameliorates sodium chloride stress on tomato plants. J. Plant. Inter. 5(1):51-58.

Thompson DC (1996). Evaluation of bacterial antagonist for reduction of summer patch symptoms in Kentucky blue grass. Plant Disease. 80:856-862.

Nellemann C, MacDevette M, Manders T, Eickhout B, Svihus B, Prins A, Kaltenborn B. (eds) (2009). The Environmental Food Crisis. The environment's role in averting future food crises. A UNEP rapid response assessment. Arendal, UNDP.

Upadhyay SK, Singh DP, Saikia R (2009). Genetic diversity of plant growth promoting rhizobacteria isolated from rhizospheric soil of

wheat under saline condition. Curr. Microbiol. 59:489 496.
Usha RM, Arundhathi, Reddy G (2011). *Bacillus cereus* and *Enterobacter cancerogenus* screened for their efficient plant growth promoting traits rhizobacteria (PGPR) and antagonistic traits among sixteen bacterial isolates from rhizospheric soils of pigeon pea. A. J. Microbial. R. 5(15):2090-2094.
Usha RM, Arundhathi, Reddy G (2012). Screening of rhizobacteria containing plant growth promoting (PGPR) traits in rhizosphere soils and their role in enhancing growth of pigeon pea. A. J. Biotechnol. 11(32):8085-8091.
Varu DK, Barad AV (2010). Standardization of mango rootstock for mitigating salt stress. Indian J. Hort. 67:79-83.
Xie H, Pasternak JJ, Glick BR (1996). Isolation and characterization of mutants of plant growth promoting rhizobacterium *Pseudomonas putida* GR 12-2 that over produce indoleacetic acid. Curr. Microbiol. 32:67-71.

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Full Length Research Paper

Pesticides jiggling microbial transformation of phosphorus in soil

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A laboratory based pot culture experiment was conducted to investigate the impact of pesticides [endosulfan, dithan-M-45 and 2,4-dichlorophenoxy acetic acid (2,4-D)] at their recommended doses on the changes in microbial biomass carbon; potentiality and proliferation of phosphorus solubilizing organisms; acid and alkaline phosphatase activities; total and available phosphorus in soil. The results of the investigation reveal that though each of the pesticides exerted deleterious effect on phosphorus solubilizing power of soil in the beginning, the overall impact was stimulatory rather than detrimental to the growth and activity of microorganisms associated with the phosphorus transformation in soil. Application of 2,4-D affected the highest level of microbial biomass carbon, growth and activity of phosphorus solubilizing microorganisms and phosphatase activity which was translated to the highest level of available phosphorus in soil. On the other hand, endosulfan brought about the least increment in the level of microbial biomass carbon; potentiality and proliferation of phosphorus solubilizing organisms; acid and alkaline phosphatase activities besides total and available phosphorus in soil. The results of the investigation reveal the safe use of each of the pesticides in the field for the eradication of pest, especially 2,4-D.

Key words: Pesticides, microbial biomass carbon, growth and activity of phosphorus solubilizing microorganisms, p-solubilizing power, phosphatases, total and available phosphate.

INTRODUCTION

Pesticides are chemicals that wipe out the biological agents responsible for the loss of economic crops. Under the umbrella of pesticides viz. insecticides, fungicides and herbicides are designed to control insects, fungi and weeds, respectively. The ultimate destination of pesticides applied directly or as spray on the foliage is the soil ecosystem comprising inanimate and living components. Soil ecosystem gets distributed by the introduction of pesticides.

Pesticides sometimes exert detrimental influence on the growth and activity of microorganisms involved in various biochemical transformations in soil which results in microbial population decline (Yaduraju et al., 2006), but, some microorganisms, having the capacity to withstand the detrimental influence of pesticides, survive. The population of those organisms rises by deriving energy and nutrients from the dead cells generated by the action of pesticides (Giri et al., 2006).

On the other hand, there are pesticides exerting stimulation (Samanta et al., 2005) by serving as nutrient and energy sources for the microorganisms. So, the population density of pesticide utilizers increases so long as source of energy and nutrients are there with a concomitant reduction in the active ingredients of pesticide

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residue (Debnath et al.,2002; Nongthombam et al., 2008). Phosphorus transformation in soil is very important, as it is one of the major essential nutrients. But, soil phosphorus is predominantly insoluble and so unavailable to plants. Plants only make use of 10 to 12 per cent of the applied phosphatic fertilizers. The rest become unavailable through biotic and abiotic process. The unavailable inorganic forms of phosphorus are tri-calcium phosphate in addition to iron and aluminium phosphates depending upon soil pH (Hue et al., 1986; Khan et al., 2009). Although, there are microbes capable of solubilizing insoluble inorganic compounds in soil. Among them, the important ones are *Pseudomonus, Bacillus, Micrococcus, Mycobacterium, Penicillium, Fusarium* and *Aspergillus* (Gaur, 2006). They make a large portion of the insoluble inorganic phosphorus soluble and the quantities in excess of their nutritional demand become available for the utilization of plants. Many of them are also capable of mineralizing organic phosphorus compounds by elaborating the enzymes-phosphatases and phytases (Tarafdar et al., 2002; Aseri et al., 2009; Ramesh et al., 2011). Crops also bring out soil enzymes in order to meet their nutritional demand.

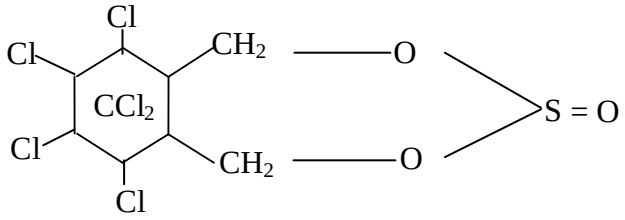
There exists conflicting reports regarding the effects of pesticides on the growth and potentiality of phosphorus solubilizing microorganisms in soil. Das et al. (2012) found a significant enhancement in the potentiality and growth of phosphate solubilizing microorganisms by the application of pesticides in soil. On the other hand, there are reports about the detrimental influence of pesticides on the growth and performance of phosphorus solubilizers (Das et al., 1998). Bibliographical antecedence speaks about the differential influence of pesticides on the mineralization of organic phosphorus in soil. In this connection, Brown and Lean (1995) found the negative aspect of pesticides on the mineralization of organic phosphorus in soil, while Das and Mukherjee (1994) observed the positive aspect, with an increase in extractable phosphorus in soil. Chemo-organotropic microorganisms through enzymes mediate the process of organic phosphorus mineralization. The contribution of acid and alkaline phosphatase is worth mentioning in this respect. However, pesticides exert differential influence on acid and alkaline phosphatase activity in soil. A group of workers advocate positive effect (Tu, 1992; Rahmansyah et al., 2009). On the contrary, others put forward negative effect (Abul et al., 2002; Jastrzebska, 2011). The above revelation reflects a great diversity in phosphorus transformation under the influence of pesticides in soil. Hence, an attempt was made to investigate the effect of pesticides on some chemical, biochemical and microbiological properties in relation to phosphorus transformations in soil.

MATERIALS AND METHODS

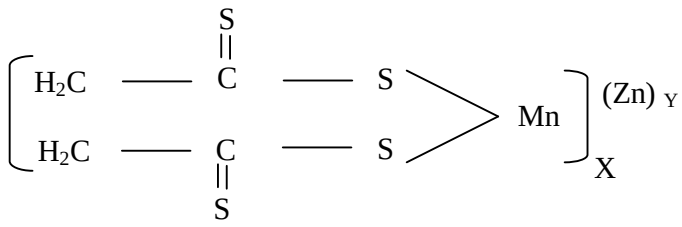
The investigation was carried out with earthen pots in the laboratory of the Department of Agricultural Chemistry and Soil Science, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, West Bengal, India.

Description of pesticides

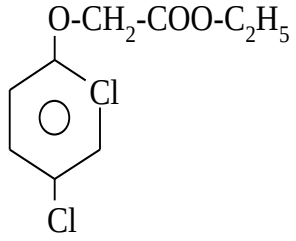
Endosulfan (Insecticide): IUPAC name- 1,2,3,4,7,7-hexachloro-bicycle(2,2,1)-heptane(2)-bis (oxymethylene)- 5,6 sulphite. Molecular name- $C_9H_6O_3Cl_6S$.



Dithane M - 45 (Fungicide): IUPAC name- Manganese-zinc, double salt of N-N-ethylene bis dithiocarbamic acid.



2, 4-D ethyl ester (Herbicide): IUPAC name- 2,4-dichlorophenoxy acetic acid. Molecular formula: $C_{10}H_{12}Cl_2O_3$.



Collection of soil

Typical gangetic alluvium (inceptisol) soil was collected from the University Instructional Farm. Surface soil samples (0-15 cm depth) gathered from monoculture (Kharif rice) cultivated field were air dried and passed through 80 mesh sieve. The physico-chemical and microbiological properties of soil are delineated hereunder in Table 1.

Experimental

100 g of soil was thoroughly mixed with urea, single super phosphate and muriate of potash at the rate of 100 Kg N, 50 Kg P_2O_5 and 50 kg K_2O ha^{-1}, respectively and placed in small earthen pots. Three pots were kept as such to be considered as triplicate control (without any pesticide). In the rest pots, each of the three pesticides endosulfan (35 EC) at 2.0 kg a.i. ha^{-1}; dithane-M 45 at 1.5 kg a.i. ha^{-1} and 2,4-D (38 EC) at 1.0 kg a.i. ha^{-1}) were again

Table 1. Physico-chemical and microbiological properties of soil.

Soil characteristic	Status
Type of soil	Alluvial
Soil taxonomy	Typic *haplustepts*
Coarse sand (%)	44.5
Fine sand (%)	20.4
Silt (%)	16.8
Clay (%)	18.2
Soil p^H (1: 2.5 w/v) in water	6.8
Organic carbon (g kg^{-1})	7.1
Total nitrogen (g kg^{-1})	0.7
Available phosphorus (mg kg^{-1})	11.7
Available potassium (mg kg^{-1})	44.0
Microbial biomass carbon (µg g^{-1})	138.2
Phosphorus solubilizers (cfu $\times$ $10^5 g^{-1}$ dry soil)	15.2
Total Phosphorus (Kg P_2O_5 ha^{-1})	536.7
Phosphate solubilizing power (mg 15 mg^{-1} insoluble P)	0.4
Acid phosphatase activity (nKat 100 g^{-1} soil)	7.4
Alkaline phosphatase activity (nKat 100 g^{-1} soil)	13.5

blended separately at three replications. The moisture level of soil for each pot was adjusted to 50% of water holding capacity of soil by compensating the loss in weight with deionized water on every alternate day. Thereafter, each pot was incubated at 37 ± 1°C for 90 days.

Sample collection and analysis

Following the method of Das et al. (2012), soil samples were collected from each of the respective pot on the 5^{th}, 10^{th}, 15^{th}, 30^{th}, 60^{th} and 90^{th} days of incubation. Soil moisture was measured immediately before the analysis of microbiological, biochemical and chemical transformations.

Microbial biomass carbon was determined by chloroform fumigation following the method of Joergensen (1995). There were two sets of moist soil- one set in conical flasks fitted with a stopper and the other set in beakers. The beakers containing soils were subjected to chloroform fumigation till the chloroform boiled in vacuum desiccator for two minutes. The desiccator was then incubated at 25°C for 24 h. Both the fumigated and non-fumigated soils were extracted with 0.5M K_2SO_4 and the extracts were analysed by potassium dichromate oxidation method (Jenkinson and Powlson, 1976). The difference in the status of organic carbon between the fumigated and non-fumigated soils, divided by a calibration factor (K_{ec} = 0.38) represented the measure of microbial biomass carbon in soil which was expressed as µg microbial biomass C g^{-1} dry soil.

Colony forming units (cfu) of phosphorus solubilizing microorganisms were enumerated by serial dilution and pour plate technique (Pramer and Schmidt, 1965) in Pikovskaia medium (1948). Phosphorus solubilizing power was determined by the estimation of soluble P in 15ml of sucrose tri-calcium phosphate broth containing 1% sucrose after incubation of 1 gm soil in culture tubes at 30±1°C for 15 days (Das and Mukherjee, 2000). Acid and alkaline phosphatase activities were determined following the procedure of Tabatabai and Bremner (1969).

Total phosphorus in soil was extracted by Bowman (1988) and estimated following modified ascorbic acid method (Dick and Tabatabai, 1977). Water-soluble phosphorus in soil was extracted in sodium bicarbonate (Jackson, 1973) and estimated through Olsen and Dean (1982).

Statistical analysis

The results were adjudicated by analysis of variance (ANOVA), and the statistical significance (P=0.05) of difference between means within factors (pesticides and incubation time) was determined by using completely randomized design following the method of Gomez and Gomez (1984).

RESULTS AND DISCUSSION

Microbial biomass carbon (MBC)

Application of pesticides- endosulfan, dithane M-45 and 2,4-D- resulted in a significant boost in the level of microbial biomass carbon over that of untreated control in soil from 5^{th} to 90^{th} days of incubation (Table 2). The extent of increment, however, differed depending on the type of pesticide. The herbicide 2,4-D generated MBC to the highest level (El-Ghamry et al., 2001) ranging from 2.7 to 5.9% over that of untreated control; while the minimum increment varying from 0.6 to 2.6% was brought about by endosulfan. However, dithane M-45 affected the increment ranging from 0.4 to 3.2% over that of control. The hike in MBC might be due to the utilization of pesticides as energy and nutrient sources by microbial community for MBC synthesis (Kumar et al., 2012). In addition, the pesticides could impose resistant microbial community through mutation (Daly et al., 1998) to spread their generations at the expense of dead susceptible organisms through energy and nutrient sources for additional microbial biomass carbon synthesis.

Table 2. Effect of pesticides on microbial biomass carbon (μg g^{-1} soil) content of the soil during different days of incubation.

Treatment	Days of incubation						Mean
	5	10	15	30	60	90	
Control	130.43±0.02	123.43±0.03	121.63±0.04	105.68±0.0058	99.28±0.01	95.79±0.02	112.71
Endosulfan	132.91±0.0058	124.08±0.0058	123.69±0.01	108.48±0.0058	100.29±0.01	97.63±0.01	114.52
Dithane-M-45	134.62±0.01	126.08±0.04	124.15±0.02	108.98±0.03	99.78±0.0058	96.24±0.0058	114.98
2,4-D	138.15±0.0058	128.63±0.0058	124.86±0.5777	113.6±0.05	103.79±0.1	100.11±0.02	118.19
Mean	134.03	125.57	123.58	109.19	100.78	97.44	

Anova (p value at 0.05): Treatment (Tr) 0.082, Days (D) 0.099, Interaction (Tr X D) 0.199. Mean of three replicates ± SD.

Table 3. Effect of pesticides on the number of phosphate solubilizing micro-organisms in the soil during different days of incubation.

Treatment	Days of incubation						Mean
	5	10	15	30	60	90	
Control	32 ± 1	49 ± 3	73 ± 0.58	109 ± 1	46 ± 0.58	35 ± 0.58	57.56
Endosulfan	37 ± 1	55 ± 4	78 ± 3	113 ± 0.58	49 ± 1	41 ± 5	62.1
Dithane-M-45	55 ± 3	62 ± 1.01	84 ± 3	119 ± 0.58	56 ± 1	48 ± 0.58	70.78
2,4-D	62 ± 0.58	74 ± 3	98 ± 3	128 ± 3	62 ± 0.58	48 ± 7	78.78
Mean	46.83	60	83.33	117.25	53.42	43.25	

Anova (p value at 0.05): Treatment (Tr) 2.005, Days(D) 1.680, interaction (Tr X D) 4.113. Means of three replications ±SD.

P-solubilizing micro-organisms

The chronological enhancing influence of 2,4-D as well as dithane M-45 and to a great extent endosulfan on phosphorus solubilizing microorganisms was almost similar to that of MBC and justifies that phosphorus solubilizers are fractional constituents of microbial community activity involved in biochemical transformation of phosphorus in spite of the influence of pesticides. Interestingly, there was a positive correlation (r = 0.955) between MBC and phosphorus solubilizing microorganisms in soil (Table 3). Although, the intensity of pesticidal impact on phosphorus solubilizing microorganisms was much higher than MBC.

Moreover, there was a rhythmic periodicity in the increment of microbial community during the incubation period. 2,4-D and dithane M-45 caused gradual declination in significant (p = 0.05) rise from 93.8 to 17.4% and 71.9 to 9.2%, respectively during initial 30 days of incubation. Then, the population density of phosphorus solubilizers increased progressively from 21.7 to 37.1% and 34.8 to 37.1% by the application of herbicide and fungicide respectively in last two successive stages of sampling. This proves that phosphorus solubilizers were zymogenous (Winogradsky, 1924) organisms flourished by the availability of organic substrates and they decline gradually by reduction of the substrate; while the progressive increment was due to multiplication of the mutant ones (Kalyanasundaram and Kabita, 2012) or resistant ones propagated through the provision of energy and nutrient sources from the dead susceptible cells (Giri et al., 2006). The change was more pronounced with the insecticide - endosulfan- in bringing about a gradual decline in significant rise from 15.6% on the 5^{th} day to 6.9% on the 15^{th} day. Then, there was significant rise of P solubilizers by 17.1% over control on the 90^{th} day following non-significant influence from 30^{th} to 60^{th} day of incubation.

Acid-alkaline phosphatase activities

Acid phosphatase activity remained non-significant by the application of each pesticide in soil up to 10 days of incubation (Table 4). On the other hand, endosulfan had non-significant influence on alkaline phosphatase activity from the beginning up to 30 days of incubation (Table 5). The non-significant influence pointed out the stationary phase or adaptive phase for the elaboration of the phosphatase enzyme. Then concurrent with the growth of phosphorus solubilizing microorganisms, pesticides, in general, and the herbicide, in particular, resulted in a significant boost in acid phosphatase activity over that of control in soil from 15^{th} to 90^{th} day of incubation; while dithane M-45 and 2,4-D caused a significant rise in alkaline phosphatase activity from 5^{th} to 90^{th} day of

Table 4. Effect of pesticides on the activity of acid phosphatase enzyme in the soil (nKat) during different days of incubation.

Treatment	Days of incubation						Mean
	5	10	15	30	60	90	
Control	7.90 ± 0.02	8.12 ± 0.026	9.09 ± 0.0057	9.92 ± 0.015	10.98 ± 0.06	11.20 ± 0.006	9.54
Endosulfan	7.90 ± 0.006	8.12 ± 0.01	9.22 ± 0.095	9.99 ± 0.0152	11.41 ± 0.01	11.78 ± 0.175	9.74
Dithane-M-45	7.91 ± 0.006	8.13 ± 0.015	9.41 ± 0.025	10.00 ± 0.021	11.01 ± 0.04	11.31 ± 0.502	9.63
2,4-D	7.92 ± 0.015	8.15 ± 0.055	9.5 ± 0.006	10.93 ± 0.072	12.92 ± 0.01	13.16 ± 0.01	10.43
Mean	7.91	8.13	9.3	10.21	11.58	11.86	

Anova (p value at 0.05): Treatment (Tr) 0.037, Days (D) 0.031, Interaction (Tr X D) 0.074. Mean of three replicates ± SD.

Table 5. Effect of pesticides on the activity of alkaline phosphatase enzyme in the soil (nKat) during different days of incubation.

Treatment	Days of incubation						Mean
	5	10	15	30	60	90	
Control	11.64 ± 0.06	11.82 ± 0.006	12.03 ± 0.015	13.91 ± 0.006	16.41 ± 0.006	17.03 ± 0.006	13.81
Endosulfan	11.88 ± 0.06	12.03 ± 0.006	12.24 ± 0.1	14.12 ± 0.01	17.00 ± 0.012	17.43 ± 0.04	14.12
Dithane-M-45	12.04 ± 0.05	12.53 ± 0.513	13.68 ± 0.02	16.43 ± 0.01	18.47 ± 0.05	19.87 ± 0.017	15.5
2,4-D	12.3 ± 0.006	12.8 ± 0.01	14.5 ± 0.01	16.39 ± 0.006	18.31 ± 0.01	19.21 ± 0.006	15.59
Mean	11.97	12.3	13.11	15.21	17.55	18.39	

Anova (p value at 0.05): Treatment (Tr) 0.102, Days (D) 0.125, Interaction (Tr X D) 0.253. Mean of three replicates ± SD.

incubation and endosulfan only on the 60th day of incubation. This may unfold the truth that phosphorus solubilzing microorganisms not only solubilize insoluble inorganic phosphorus compounds but also mineralize organic phosphorus through acid and alkaline phosphatase activities (Khan et al., 2009). However, 2,4-D caused a progressive increment from 4.5% on the 15th day to 17.7% on the 60th day and then 17.5% on the 90th day as compared to that of control. The corresponding figures for endosulfan on the 15th and then from 60th to 90th day were 1.4% and 3.9 to 5.2% respectively while those for dithane M-45 were 3.5% and 0.8 to 1.0%, respectively. In the case of alkaline phosphatase activity, 2,4-D resulted in a significant increase ($p = 0.05$) from 5.7% on the 5th day to 20.5% on the 15th day; then a gradual reduction up to 11.6% on the 60th day followed by 12.8% increase on the 90th day over that of untreated control.

On the other hand, dithane M-45 resulted in a significant progressive rise from 3.4% on the 5th day to 18.1% on the 30th day; then a diminution by 12.6% on the 60th day followed by an increase of 16.7% on the 90th day. However, endosulfan resulted in a significant increment of 3.6% in alkaline phosphates activity only on the 60th day as compared to that of control.

Phosphorus solubilizing power

Each of the pesticides induced an initial significant detrimental influence on the potentiality of phosphorus solubilizing microorganisms in soil up to 10th day of incubation as compared to that of control (Koley and Dey, 1989). The ferocity of pesticidal impact on phosphorus solubilizing power, though declined from 5th to 10th day, was most fierce (55.3 to 45.3%) in endosulfan followed by dithane M-45 (48.9 to 41.5%) and 2,4-D (40.4 to 32.1%) respectively as compared to that of untreated control (Table 6). The impaired effect was due to greater utilization of solubilized insoluble phosphate by phosphate solubilizers than that of their capacity of solubilization of tricalcium phosphate. This is also an indication of higher induction on multiplication than the efficiency of phosphorus solubilizers by the pesticides in soil (Das et al., 1998).

However, the harmful effect did not last long and similar to the proliferation of P-solubilizing microorganisms, application of the herbicide and fungicide exerted significant stimulation on phosphorus solubilizing capacity of soil from 15th to 90th day of incubation which, in turns, gradually reduced from 110% and 80 to 9.1 and 10.2%, respectively, as compared to that of untreated control in soil (Table 6). Endosulfan also significantly enhanced the efficiency of phosphorus solubilizing organisms by 43.3 and 8.6% on the 15th and 60th day, respectively over that of control (Kukreja et al., 2010). A cursory glance at the results reveal that though pesticides brought about gruesome influence in the beginning on phosphorus solubilizing power in soil, the horror subsided very soon and in accordance with the chronological sequence of phosphate solubilizers. 2,4-D augmented the efficiency of

Table 6. Effect of pesticides on phosphate solubilising capacity of the soil (mg/15 g insoluble P per g soil per 0.15g sucrose) during different days of incubation.

Treatment	Days of incubation						Mean
	5	10	15	30	60	90	
Control	0.047 ± 0.0006	0.053 ± 0.004	0.03 ± 0.0006	0.07 ± 0.03	0.081 ± 0.002	0.088 ± 0.005	0.062
Endosulfan	0.021 ± 0.01	0.029 ± 0.002	0.043 ± 0.0006	0.07 ± 0.006	0.088 ± 0.01	0.089 ± 0.0006	0.057
Dithane-M-45	0.024 ± 0.003	0.031 ± 0.0006	0.054 ± 0.0006	0.082 ± 0.0006	0.094 ± 0.003	0.097 ± 0.002	0.064
2,4-D	0.028 ± 0.002	0.036 ± 0.005	0.063 ± 0.007	0.088 ± 0.02	0.092 ± 0.001	0.096 ± 0.0006	0.067
Mean	0.03	0.037	0.048	0.077	0.089	0.093	

Anova (p value at 0.05): Treatment (Tr) 0.0028, Days (D) 0.0028, Interaction (Tr X D) 0.0057. Mean of three replicates ± SD.

Table 7. Effect of pesticides on the level of total phosphorus in soil (Kg P_2O_5 ha^{-1}).

Treatment	Days of incubation						Mean
	5	10	15	30	60	90	
Control	534.00 ± 2.65	530.67 ± 0.58	529.67 ± 1.15	528.00 ± 1.0	525.33 ± 1.53	523.67 ± 5.69	528.56
Endosulfan	535.33 ± 0.58	532.67 ± 0.58	530.67 ± 1.15	529.00 ± 0.01	526.00 ± 1.0	522.33 ± 1.15	529.33
Dithane-M-45	536.00 ± 4.0	533.00 ± 3.0	530.67 ± 0.58	529.67 ± 0.58	526.67 ± 1.53	524.33 ± 0.58	530.06
2,4-D	536.67 ± 0.58	534.00 ± 1.0	532.33 ± 3.21	530.67 ± 0.58	527.33 ± 2.08	524.67 ± 1.53	530.94

Anova (p value at 0.05): Treatment (Tr) 1.339, days (D) 1.637, interaction (Tr X D) NS. Mean of three replicates ± SD.

Table 8. Effect of pesticides on the available phosphorus content ($mg.kg^{-1}$) in the soil during different days of incubation.

Treatment	Days of incubation						Mean
	5	10	15	30	60	90	
Control	72.34 ± 0.07	81.3 ± 0.006	89.25 ± 0.01	103.53 ± 0.021	98.61 ± 0.051	71.43 ± 0.012	86.08
Endosulfan	74.39 ± 0.053	82.6 ± 0.006	90.45 ± 0.006	105.51 ± 0.10	99.72 ± 0.08	74.36 ± 0.04	87.84
Dithane-M-45	75.1 ± 0	83.11 ± 0.01	92.12 ± 0.02	112.66 ± 0.36	100.83 ± 0.125	75.00 ± 0.017	89.80
2,4-D	76.33 ± 0.006	85.12 ± 0.04	94.23 ± 0.006	116.07 ± 0.071	102.11 ± 0.12	78.13 ± 0.006	92.00
Mean	74.54	83.03	91.15	109.44	100.32	74.73	

Anova (p value at 0.05): Treatment (Tr) 0.060, days (D) 0.073, interaction (Tr X D) 0.148. Mean of three replicates ± SD.

phosphorus solubilizers to the highest extent followed by dithane M-45 and endosulfan, respectively.

Total and available phosphorus

Application of insecticide, fungicide and herbicide did not render any significant influence on the level of total phosphorus in soil as compared to that of control nor was there significant difference among the pesticides at any stage of sampling (Table 7). The results thus reflect virtually negligible loss of total phosphorus by biotic or abiotic means. However, each of the pesticides resulted in a significant enhancement in the level of available phosphorus over that of control from the 5^{th} to 90^{th} day of incubation in soil (Table 8). 2,4-D caused significant ($p = 0.05$) increase ranging from 4.7% to 10.0% in between 5^{th} to 90^{th} day of incubation over that of control. The corresponding figures for dithane M-45 and endosulfan were 2.2 to 6.8% and 1.1 to 4.1%, respectively. Using pesticides as nutrient and energy source (Paul et al., 2010), the higher growth and activity of P-solubilizing micro-organisms as well as greater acid and alkaline phosphatase activity affected greater solubilization of insoluble inorganic phosphate compounds as well as mineralization of organic P to a larger extent. This in turn was translated to the enhancement in P availability of soil (Das and Debnath, 2006; Das et al., 2012). Even the microbial phosphorus may provide addendum to P availability in soil after mineralization of dead zymogenous organisms (Tiessen et al., 2011).

Each of the pesticide- 2,4-D, dithane M-45 and endosulfan- at their field recommended doses caused a significant acceleration in solubilization and mineraliza-

tion of insoluble inorganic and organic P compounds in spite of detrimental influence on the potentiality of P-solubilizing micro-organisms in the beginning. As a consequence, there was higher retention of available P in soil. So, all of the pesticides are not only safe but also rejuvenator of mobile P in soil ecosystem. Among the pesticides, 2,4-D performance is preeminent in all virtues.

REFERENCES

Abul K, Mukherjee AK, Kalam A (2002). Effects of hexaconazole, tropiconazole, profenofos and pretilachlor on soil phosphatase and dehydrogenase activities. Pestic. Res. J. 14:337-342.

Aseri GK, Jain N, Tarafdar JC (2009). Hydrolysis of Organic Phosphate Forms by Phosphatases and Phytase Producing Fungi of Arid and Semi Arid Soils of India. Am. Eurasian J. Agric. Environ. Sci. 5:564-570.

Bowman RA (1988). A rapid method to determine total phosphorus in soils. Soil Sci. Soc. Am. J. 52:1301-1304.

Brown LS, Lean DRS (1995). Toxicity of selected pesticides to lake phytoplankton measured using photosynthetic inhibition compared to maximal uptake rates of phosphate and ammonium. Environ. Toxicol. Chem. 14:93-98.

Daly H, Doyen JT, Purcell AH III (1998). Introduction to insect biology and diversity, 2nd edition. Oxford University Press. New York.

Das AC, Debnath A (2006). Effect of systemic herbicides on N_2 fixing and phosphate solubilizing microorganisms in relation to availability of nitrogen and phosphorus in paddy soils of West Bengal. Chemosphere 65:1082-1086.

Das AC, Mukherjee D (1994). Effect of insecticides on the availability of nutrients, nitrogen fixing and phosphate solubility in the rhizosphere soil of rice. Biol. Fertil. Soils 18:37-41.

Das AC, Mukherjee D (2000). Influence of insecticides on microbial transformation of nitrogen and phosphorus in Typic Orchragualf soil. J. Agric. Food Chem. 48:3728-3732.

Das AC, Nayek H, Chakravarty A (2012). Soil application of dinitroaniline and arylphenoxy propionic herbicides influences the activities of phosphate solubilising micro-organisms in soil. Environ. Monit. Assess. 184:7453-7459.

Das AC, Saha SK, Chakravarty A, Mukherjee D (1998). Effect of Insecticides on the microbial populations and their biochemical activities as influenced by organic matter in a Typic fluvaquent soil. Indian Agric. 42:1-12.

Debnath A, Das AC, Mukherjee D (2002). Rhizosphere effect of herbicides on nitrogen fixing bacteria in relation to availability of nitrogen in rice soil. J. Indian Soc. Soil Sci. 50:463-466.

Dick WA, Tabatabai MA (1977). Determination of orthophosphate in aqueous solutions containing labile organic and inorganic phosphorus compounds. J. Environ. Qual. 6:82-85.

El-Ghamry AM, Huang CY, Xu JM (2001). Combined effect of two sulfonylurea herbicides on soil microbial biomass and N mineralization. J. Environ. Sci. 13:311-317.

Gaur AC (2006). Biofertilizers in Sustainable Agriculture. Indian Council of Agricultural Research. New Delhi.

Giri PK, Samanta A, Das S, Ghosh T, Halder M, Mukherjee D (2006). Persistance of pesticides on the proliferation and potentialities of nitrogen fixing micro-organisms in soil. J. Interac. 10:349 - 353.

Gomez KA, Gomez AA (1984). Statistical procedures for Agricultural Research. John Wiley and sons. New York.

Hue NV, Craddock GR, Adams F (1986). Effect of organic acids on aluminium toxicity in subsoils. J. Soil Sci. Soc. Am. 50:28-34.

Jackson ML (1973). Soil chemical analysis. Prentic Hall of India Pvt. Ltd., New Delhi.

Jastrzebska E (2011). The effect of Chloropyriphos and Teflubenzuron on the enzymatic activities of soil. Pol. J. Environ. Stress 20:903-910.

Jenkinson DS, Powlson DS (1976). The effects of biocidal treatments on metabolism in soil. Fumigation with chloroform. Soil Biol. Biochem. 8:167-177.

Joergensen RG (1995). Microbial biomass. In: methods in applied microbiology and biochemistry (Alef, K and Nannipieri, P., eds). Academic press.

Kalyanasundaram D, Kavitha S (2012). Effect of Butachlor on the Microbial Population of Direct Sown Rice. World Academy of Science, Engineering and Technology 69:853-855.

Khan AA, Jilani G, Akhtar MS, Naqvi SMS, Rasheed R (2009). Phosphorus Solubilizing acteria: Occurrence, Mechanisms and their Role in Crop Production. J. Agric. Biol. Sci. 1:48-58.

Koley SC, Dey BK (1989). Effect of aromatic amine herbicides on microbial population and phosphate solubilizing power of the rhizosphere soil of groundnut, Indian Agric. 33:1-8.

Kukreja GP, Bhute SS, Mangate SA, Dhawale MN (2010). Exploring the Potential of Species as Phosphate Solubilizer,Plant Growth Promoter, Biocontrol Agent and Pesticide Degrader. Asian J. Exp. Biol. Sci. 40-44.

Kumar A, Nayak AK, Shukla AK, Panda BB, Raja R, Shahid M, Tripathi R, Mohanty S, Rath PC (2012). Microbial Biomass and Carbon Mineralization in Agricultural Soils as Affected by Pesticide Addition. Bull. Environ. Contam. Toxic. 88:538-542.

Nongthombam S, Nayek H, Das AC (2008). Effect of anilofos and pendimethalin herbicides on N_2-fixing and phosphate solubilizing microorganisms in relation to availability of nitrogen and phosphorus in a Typic Haplustept soil. J. Crop Weed 4:1-6.

Olsen SR, Dean LA (1982). Phosphorus. In C.A Black, D.P. Evans, J.L. White, L.E. Ensminger, F.E. Clark, & R.C. Diahnauer (Eds.) Methods of soil analysis. Madison: American Society of Agronomy. pp. 1035-1049.

Paul N, Ghosh M, Das DK, Sur P, Mukherjee D (2010). Pesticidal impact on DTPA extractable Iron vis-à-vis actinomycetes in soil. India Agric. 54(1&2):83-86

Pikovskaia RI (1948). Mobilization of phosphates in soil in connection with the vital activation of some microbial species. Microbial Biol. 17: 362-370.

Pramer D, Schmidt EL (1965). Experimental Soil microbiology, Burges Publishing Co. Minneapolis. 5, Minn.

Rahmansyah M, Antonius S, Sulistinah N (2009). Phosphatase and urease instability caused by pesticides present in soil improved by grounded rice straw. ARPN J. Agric. Biol. Sci. 4:56-62.

Ramesh A, Sharma SK, Joshi OP, Khan IR (2011). Phytase, Phosphatase Activity and P-Nutrition of Soybean as Influenced by Inoculation of Bacillus. Ind. J. Microbiol. 51:94-99.

Samanta A, Ghosh S, Haldar M, Mukherjee D, Ghosh RK (2005). Influence of herbicides on microorganisms and transformation of iron in rice rhizosphere soil. J. Inter. 9:528-532.

Tabatabai MA, Bremner JM (1969). Use of P-nitrophenyl phosphate for assay of soil phosphatise activity. Soil Biol. Biochem. 1:301-307.

Tarafdar JC, Yadav RS, Niwas R (2002). Relative efficiency of fungal intra- and extracellular phosphatase and phytase. J. Plant Nutr. Soil Sci. 165:17-19.

Tiessen H, Ballester MV, Salcedo I (2011) Phosphorus and global change. In: Bunemann EK,Oberson A, Frossard E (eds) Phosphorus in action: biological processes in soil phosphorus cycling. Soil Biol. 26:9-18.

Tu CM (1992). Effect of three newer pesticides on microbial and enzymatic activites of soil. Bull. Environ. Contam. Toxic. 49:120-128.

Winogradsky S (1924). Sur la microflora autochtone de la terre arable. Compt. Rend. Acad. Sci. 178:1236-1239.

Yaduraju NT (2006). Herbicide resistant crops in weed management. In: The Extended Summaries, Golden Jubilee National Symposium on Conservation Agriculture and Environment. Octo., 26-28, Banaras Hindu University, Banaras. pp. 297-98.

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Combined activity of garlic and nitrofurantoin against *Escherichia coli* and *Enterococcus* species recovered from urinary tract infections

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Nitrofurantoin represents an attractive choice for empirical treatment of acute uncomplicated cystitis, and for long term prophylaxis against recurrent urinary tract infections. However, rather rare but severe adverse effects related to cumulative drug doses may occur. Bioactive compounds of plant origin combined with antibiotics can increase the sensitivity of microbial cells to such antibiotics. Garlic has antimicrobial effects against a wide range of microorganisms. The minimum inhibitory concentrations (MICs) of each of nitrofurantoin and garlic alone, and in combination were determined against 17 extended spectrum β-lactamase (ESBL) producing *Escherichia coli* and 24 *Enterococcus* spp. urinary isolates. When grown as planktonic cells, none of the *E. coli* isolates demonstrated resistance towards nitrofurantoin, whereas only one (4.2%) *Enterococcus* isolate was resistant. Garlic showed an inhibitory effect on planktonically grown ESBL producing *E. coli* and *Enterococcus* spp. with varying MICs. Each of nitrofurantoin and garlic tested alone showed an increase in the MICs for biofilm grown isolates compared to their planktonic counterparts. However, the combination of both agents led to significant decline of the MICs, whether for planktonic or biofilm forms, resulting in either synergy or addition. In conclusion, garlic enhanced the antibacterial activity of nitrofurantoin towards the tested urinary isolates.

Key words: Antimicrobial combination, biofilm, checkerboard assay, *Enterococcus* spp., extended spectrum β-lactamase (ESBL)-producing *Escherichia coli,* garlic extract, nitrofurantoin.

INTRODUCTION

Urinary tract infections (UTIs) are the commonest encountered in clinical practice (Butt et al., 2004), and many of the causative micro-organisms tend to respond to the urinary tract environment by biofilm formation producing chronic and often intractable infections (Subramanian et al., 2012). *Escherichia coli* is the most predominant pathogen causing community and nosocomially-acquired UTIs (Ejrnæs, 2011); which has become difficult to treat due to the increased prevalence of extended-spectrum β-lactamase (ESBL) production (Chaudhary and Aggarwal, 2004). On the other hand, UTIs due to Gram-positive bacteria are fairly uncommon; however they are usually caused by *Staphylococcus* spp. and enterococci (Bouza et al., 2001; Baral et al., 2013). Multi-drug resistance is very common among urinary enterococci, resulting in lack of oral treatment alternatives (Baral et al., 2013).

Nitrofurantoin is a synthetic antibacterial agent effective against most common Gram-negative and Gram-positive urinary tract pathogenic bacteria (Rafii and Hansen, 1998). The drug's susceptibility correlates with the reduction of

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nitrofurantoin inside the bacterial cell to highly reactive intermediates that attack the cell's ribosomal proteins, DNA and other macromolecules (Cetti et al., 2009). Fortunately, resistance to this drug has remained virtually low and unchanged since its introduction in 1953, making it an attractive choice for empirical treatment of acute uncomplicated cystitis (Gupta et al., 2011) and long term prophylaxis against recurrent UTIs (Karpman and Kurzrock, 2004; Cetti et al., 2009). However, severe but rather rare adverse reactions such as hepatotoxicity and neuropathy based on cumulative doses (Karpman and Kurzrock, 2004; Sharafadinzadeh et al., 2008) necessitate caution when issuing nitrofurantoin prescriptions and a need for changing the management protocols (Cetti et al., 2009).

Garlic (*Allium sativum*), an essential food ingredient worldwide, has long been known to have antibacterial, antifungal and antiviral effects. The main antimicrobial constituent of garlic, allicin, is generated by the enzyme alliinase when garlic is crushed (Lee et al., 2011). Allicin interacts with important thiol-containing enzymes as cysteine proteinases, alcohol dehydrogenase, as well as the thioredoxin reductases, which are critical for maintaining the correct redox state within microorganisms (Ankri and Mirelman, 1999). Enhancement of the antibacterial activity of different antibiotic classes towards Gram-positive species, Gram-negative species and even *Mycobacterium tuberculosis* has been demonstrated in various combination studies with either crude garlic extract or allicin (Ankri and Mirelman, 1999; Jonkers et al., 1999; Cai et al., 2007). Relatively few side effects have been reported in humans using garlic and its preparations, mostly related to gastrointestinal discomfort and nausea (Banerjee and Maulik, 2002). On the other hand, high garlic concentrations have shown to be clastogenic in mice (Das et al., 1996), and have induced altered structure and function of the heart, liver and kidneys in rats when administered for prolonged periods (Banerjee and Maulik, 2002).

The aim of the present study was to examine the *in vitro* activity of garlic and its potential for synergy when combined with nitrofurantoin against urinary isolates of *E. coli* and enterococci.

MATERIAL AND METHODS

Bacterial isolates

The present study was conducted on non-duplicate urinary isolates belonging to *E. coli* and *Enterococcus* spp. Seventeen ESBL producing *E. coli* isolates recovered from UTIs and identified conventionally (Nataro et al., 2011) were included in the study. ESBL-expression was confirmed by the double disk synergy test according to the clinical laboratory standards institute (CLSI) document M100-S23 (CLSI, 2013). In addition, 24 enterococcal isolates which were recovered from patients with UTI and identified conventionally at first according to Teixeira et al. (2011) followed by the API-20 Strep system (Bio-Merieux,Marcy-l'Etoile, France) were included in the present study. All isolates were tested by the disc diffusion method (Patel et al., 2011) for their susceptibility towards antibiotics used for urinary tract infections, namely; lomefloxacin (10 µg), norfloxacin (10 µg) and trimethoprim-sulphamethoxazole (1.25/23.75 µg); were tested against the *E. coli* isolates, whereas ciprofloxacin (5 µg), levofloxacin (5 µg), norfloxacin (10 µg) and tetracycline (30 µg) were used for isolates of the *Enterococcus* spp. (CLSI, 2013). *E. coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 were also included in the study as quality control strains.

Biofilm production

The tissue culture plate method was adapted for quantitative detection of biofilm production according to Hassan et al. (2011) for *E. coli* and Marra et al. (2007) as well as Kouidhi et al. (2011) for enterococci. Overnight cultures from fresh agar plates were inoculated onto trypticase soy broth (Oxoid Ltd., Hampshire, England) with 1% glucose (w/v) and brain heart infusion broth (Oxoid Ltd., Hampshire, England) with 2% glucose (w/v) for *E. coli* and enterococci; respectively. After overnight incubation at 37°C, the broths were diluted 1:100 with their corresponding media. Then, 200 µl of these cell suspensions were inoculated into sterile 96 well flat bottom polystyrene tissue culture treated plates (Nunc, Roskilde, Denmark) and incubated 24 h at 37°C. Each isolate was tested in duplicates and uninoculated wells containing sterile broth served as negative control. Biofilms formed by *E. coli* were fixed by 2% sodium acetate and stained by 0.1% crystal violet, followed by removing excess stain by deionized water and drying the plates. On the other hand, enterococcal biofilms were stained by 0.2% crystal violet and washed 3 times with phosphate buffered saline (PBS), followed by extraction of the crystal violet bound to the biofilm with 200 µl of an 80:20 mixture of ethanol and acetone.

Biofilm formation was assessed spectrophotometrically using a microplate reader (Stat Fax-2100, Awareness) at 570 and 595 nm for *E. coli* and enterococci; respectively, whereby isolates were classified as non biofilm producers, weak producers or strong producers according to Christensen et al. (1985).

Garlic extraction

1 kg of garlic cloves was pulverized and soaked daily on 3 consecutive days in 500 ml ethanol at room temperature. The ethanol extract was concentrated under reduced pressure at 40°C to give a residue of 60 g. (Wu et al., 2012). The powder extract was weighed and dissolved in a measured amount of sterile distilled water to reach the desired concentration. Then, it was sterilized using Nalgene^{TM} 0.45 µm syringe filter (Thermo Fisher Scientific, Langenselbold, Germany) and the concentration of the filtered extract was considered as 50% that of the pre-filtered one (Hindi, 2013).

Determination of the antimicrobial activity

The minimum inhibitory concentration (MIC) of nitrofurantoin (Macrofuran 50 mg capsules, KahiraPharm, Egypt) for isolates of *E. coli* and *Enterococcus* spp. was determined by the broth microdilution method (CLSI, 2013). The procedure was also adapted for testing serial dilutions of freshly dissolved garlic extract. The tested final nitrofurantoin concentrations ranged from 0.5 – 256 µg/ml dissolved in sterile dimethyl sulfoxide and the MIC breakpoint interpretation was done according to the CLSI guidelines (CLSI, 2013), whereas the tested final garlic concentrations ranged initially from 97.66 to 25,000 µg/ml., followed by extra sets of concentrations ranging from 37,500 to 150,000 µg/ml and from 50,000 to 200,000 µg/ml, when growth was observed throughout all the initial concentrations.

Moreover, the susceptibility of the biofilm grown *E. coli* and enterococci isolates towards each of nitrofurantoin and garlic was done by the microplate alamar blue assay (Flemming et al., 2009). Briefly, washed biofilms were subjected to treatment with the previously mentioned agents with the same prepared concentrations. Positive controls formed of untreated biofilms with only 200 µl of Mueller-Hinton II broth (MHB) (Oxoid Ltd., Hampshire, England), and negative controls formed of only 200 µl MHB with no added bacteria were included in the assay. After incubation at 37°C for 24 h, wells were washed twice with PBS, then the metabolic activity of the biofilms was quantified by adding 250µl MHB with 5% alamar blue (AbD Serotec, Oxon, UK) per well. The plates were shaken gently for 30 s, incubated for 1 h at 37°C and the absorbance was obtained at 570 and 600 nm. Then, the alamar blue percent reduction was calculated and the minimal biofilm inhibitory concentration was defined as the lowest drug concentration resulting in ≤50% reduction and a blue well.

Assessment of the interaction between nitrofurantoin and garlic

The *in vitro* activity of nitrofurantoin and garlic in combination against each of *E. coli* and enterococci urinary isolates was tested by the checkerboard method (Isenberg, 2007).

Planktonic checkerboard assay

The range of concentrations was prepared according to the previously determined MIC of each agent for each isolate. Tested concentrations ranged from 0.03x MIC to 4.0x MIC for each agent. In addition, the MIC of each of nitrofurantoin and garlic alone was determined on different sides of the same plate. Positive controls (only bacterial inoculum and MHB) and negative controls (only MHB) were also included in each plate. The final concentration of the inoculum was 10^6 CFU/ml. After incubation at 37°C for 24 h, the combined effect was analyzed by calculation of the fractional inhibitory concentration index (FICI) using the following equation: FICI= (MIC of drug A in the combination/MIC of drug A alone)+(MIC of drug B in the combination/MIC of drug B alone). The lowest FICI value obtained in the checkerboard test was considered to be representative of the interaction of the two agents against the respective isolate. FICI results for each combination were interpreted as synergy for FICI ≤0.5, additive for 0.5 < FICI ≤ 1, indifferent for 1< FICI < 4 and antagonistic for FICI ≥ 4 (Pillai et al., 2005).

Biofilm checkerboard assay

Formed biofilms were tested by the previously described microplate alamar blue assay, except that combinations were used instead of individual agents in the same manner as the planktonic checkerboard assay. Addition of alamar blue, calculation of its percent reduction and the FIC indices as well as their interpretation was done exactly as previously described.

Statistical analysis

Data were statistically described in terms of mean ± standard deviation (± SD), median and range, or frequencies (number of cases) and percentages when appropriate. Comparison of numerical variables between the study groups was done using Mann Whitney U test for independent samples. Within each group, comparison of numerical variables was done using Wilcoxon signed rank test for paired (matched) samples. For comparing categorical data, Chi square (χ^2) test was performed. Exact test was used when the expected frequency was less than 5. *P* values less than 0.05 were considered statistically significant. All statistical calculations were done using computer programs SPSS (Statistical Package for the Social Science; SPSS Inc., released 2006. SPSS Statistics for Windows, version 15.0. Chicago, IL, USA).

RESULTS

The present study was conducted on 17 ESBL-producing *E. coli* urinary isolates, as well as 24 enterococcal urinary isolates; 21/24 (87.5%) *E. faecalis* and 3/24 (12.5%) *E. faecium*. Biofilm production was detected in 3/17 (17.6%) *E. coli* and 14/24 (58.3%) *Enterococcus* spp.; two out of which belonged to *E. faecium* and were weak biofilm forming (Table 1). *E. coli* ATCC 25922 was non-biofilm forming, whereas *E. faecalis* ATCC 29212 was strong biofilm forming.

Testing by the disc diffusion method showed that all the *E. coli* isolates (100%) were resistant to lomefloxacin and norfloxacin, whereas 14 out of the 17 isolates (82.4%) were resistant to trimethoprim-sulphamethoxazole. Concerning isolates belonging to the *Enterococcus* spp., resistance was least detected towards each of levofloxacin and norfloxacin (6/24; 25% each), followed by ciprofloxacin (7/24; 29.2%), then tetracycline (21/24; 87.5%). On the other hand, resistance towards nitrofuran-toin was not detected among the *E. coli* isolates, but was detected in only one (4.2%) *Enterococcus* isolate. The antimicrobial activity of nitrofurantoin and garlic towards the tested urinary isolates when grown planktonically *in vitro* is summarized in Table 2. The MIC of nitrofurantoin for *E. coli* ATCC 25922 and *E. faecalis* ATCC 29212 was 8 µg/ml and 4 µg/ml; respectively, whereas the garlic MIC was 12,500 and 25,000µg/ml; respectively.

There was a significant decline in the MIC values for each of nitrofurantoin and garlic when combined together against planktonic growth of all tested urinary isolates compared to their values when tested alone (Table 3). No antagonistic activity was demonstrated towards any of the tested isolates. The combination was synergistic for more *E. coli* isolates than enterococci (47% *versus* 41.7%; respectively), but with no statistical significance (*P* = 0.425). The FIC index for 50% and 90% of the *E. coli* and enterococci isolates was the same (Table 4). Combination of nitrofurantoin and garlic on E*. coli* ATCC 25922 and *E. faecalis* ATCC 29212 resulted in an FICI of 0.75 (addition) and 0.375 (synergy); respectively.

Each of nitrofurantoin and garlic tested alone against the three biofilm forming *E. coli* isolates showed an increase in the MIC values compared to their corresponding planktonic values. Nevertheless, the combination of both agents on these sessile forms led to a decline of each agents' MICs, with the result of either synergy or addition (Table 5). On the other hand, the MIC of each of nitrofurantoin and garlic when tested alone against the biofilm foming enterococci (14 isolates) exceeded 512 and 800,000 µg/ml; respectively. Accordingly, the combi-

Table 1. Biofilm production among the studied urinary isolates.

Specie	Biofilm production		
	-	+	+++
E. coli (n=17)	14 (82.4%)	1 (6%)	2 (11.8%)
***Enterococcus* spp.** (n=24)	10 (41.7%)	7 (29.2%)	7 (29.2%)

(-) = no production; (+) = weak production; (+++) = strong production.

Table 2. Antimicrobial activity of nitrofurantoin and garlic against planktonic growth of *E. coli* and *Enterococcus* spp.

Specie	Nitrofurantoin Susceptibility			Nitrofurantoin MIC (µg/ml)			Garlic MIC (µg/ml)		
	S	I	R	Range	*MIC50	**MIC90	Range	*MIC50	**MIC90
E. coli (n=17)	11 (64.7%)	6 (35.3%)	0	8 - 64	32	64	6250-100,000	25,000	100,000
Enterococcus spp. (n=24)	20 (83.3%)	3 (12.5%)	1 (4.2%)	4 - 512	8	64	25,000-75,000	25,000	37,500

S = sensitive; I = intermediate; R = resistant. *MIC of 50% of the isolates; ** MIC of 90% of the isolates.

Table 3. Mean nitrofurantoin and garlic MIC values for the tested isolates when grown planktonically.

Specie	Nitrofurantoin Mean (±SD) MIC values (µg/ml)		*P*	Garlic Mean (+SD) MIC values (µg/ml)		*P*
	Alone	In combination		Alone	In combination	
E. coli	39±20.373	13.53±8.762	*<0.001	37.8±31.1**	6.5±43.2**	*<0.001
Enterococcus spp.	37.33±103.057	10.04±25.574	*<0.001	34.3±13.1**	10.8±10.9**	*<0.001

**P* value < 0.05 is significant; ** x1000

Table 4. Effect of nitrofurantoin and garlic combination on the tested urinary isolates in planktonic growth.

Species	FICI			Effect of combination			
	Range	*FICI 50	**FICI 90	Syn	Add	Ind	Ant
E. coli (n=17)	0.25-1.25	0.625	0.75	8 (47%)	8 (47%)	1 (5.9%)	0
Enterococcus spp. (n=24)	0.375-1	0.625	0.75	10 (41.7%)	14 (58.3%)	0	0

*FICI achieved by 50% of the isolates; **FICI achieved by 90% of the isolates. Syn = Synergistic; Add = additive; Ind = indifferent; Ant = antagonistic.

nation effect of nitrofurantoin and garlic could not be assessed on the sessile growth of such isolates.

DISCUSSION

UTIs represent a major public health problem (Butt et al., 2004). Biofilm-linked infections are particularly problematic, because biofilm associated bacteria can withstand host immune defenses, antibiotics, biocides and hydrogen shear forces far better than the corresponding planktonic bacteria (Hancock et al., 2010).

In the present study, biofilm production was detected in only 3/17 (17.6%) urinary ESBL producing *E. coli* isolates. Our results are almost comparable to the results of Novais et al. (2013), where 25.7% ESBL producing urinary *E. coli* isolates produced biofilm. Higher (52%) and lower (6%) results have been reported by Ghanwate (2012) and Ponnusamy et al. (2012); respectively. *E. coli* biofilms are often associated with long term persistence of the organism in the environment (Ponnusamy et al., 2012). This has been supported by observing that *E. coli* biofilm is not a relevant virulence factor for acute cystitis but is frequently described for catheter-associated, chronic and

Table 5. Effect of nitrofurantoin and garlic alone and combined on the three biofilm forming ESBL producing *E. coli* isolates

Isolate Number	Nitrofurantoin MIC (µg/ml)			Garlic MIC (µg/ml)			Effect of combination	
	Alone on planktonic growth	Alone on biofilm	In combination on biofilm	Alone on planktonic growth	Alone on biofilm	In combination on biofilm	FICI	Result
E. coli 16	64	128	64	100,000	200,000	50,000	0.75	Add
E. coli 28	32	64	8	25,000	200,000	50,000	0.375	Syn
E. coli 37	64	2048	16	6250	200,000	50,000	0.2578	Syn

Add = Additive; Syn = Synergistic.

recurrent UTIs (Norinder et al., 2012). An issue that was not addressed in this study was whether the patients, from whom the urinary specimens were collected, were catheterized or not, or whether they were suffering from acute, chronic or recurrent UTIs.

On the other hand, 14 out of 24 (58.3%) urinary enterococcal isolates produced biofilm in the current study. Our results are in agreement with the results of others (Sandoe et al., 2003; Seno et al., 2005). A higher rate for urinary enterococcal biofilm production reaching 74% has also been reported (Comerlato et al., 2013). Biofilm production by enterococci is important in its pathogenesis (Upadhyaya et al., 2011) and is mutlifactorial depending on a number of genes working together along with external factors (Comerlato et al., 2013).

ESBL producing *E. coli* causing UTIs have been primarily considered as multi-resistant organisms originating in hospitals and have been observed in outpatient settings as well (Chaudhary and Aggarwal, 2004). In the present study, in addition to being ESBL producing, all tested *E. coli* isolates were resistant to lomefloxacin as well as norfloxacin, and 14 out of the 17 isolates (82.4%) were resistant to trimethoprim-sulphamethoxazole. However, no resistance could be detected by the planktonically grown ESBL producing *E. coli* isolates towards nitrofurantoin. Our results are comparable to the results of Auer et al. (2010) and Mukherjee et al. (2013). Also, Tasbakan et al. (2012) reported clinical and micro-biological success rates of 69% and 68%; respectively by nitrofurantoin on 75 patients with ESBL-producing *E. coli* related lower UTIs. A finding which they reported supports the suggestion that nitrofurantoin may be an alternative treatment for UTIs caused by such organisms.

Regarding isolates of the *Enterococcus* spp., our study detected resistance to nitrofurantoin in only one out of the 24 (4.2%) isolates. Comparable results have been reported by others (Chayakul et al., 2007; Karlowsky et al., 2011). On the other hand, higher resistance was detected towards other antibiotics; where 25% of the isolates were resistant to each of levofloxacin and norfloxacin, 29.2% were resistant to ciprofloxacin and 87.5% were resistant to tetracycline. Similar findings have been reported by Butt et al. (2004), who noticed that most of the nitrofurantoin susceptible enterococcal isolates were resistant to all other available antibiotics.

A wide range of microorganisms have been shown to be sensitive to crushed garlic preparations increasing the interest towards garlic as a medicinal panacea (Ankri and Mirelman, 1999). In the present study, garlic showed an inhibitory effect on ESBL producing *E. coli* with concentrations ranging from 6250 to 100,000 µg/ml and a mean of 37,867±31,130.285. Lower results have been demonstrated for commensal and pathogenic *E. coli* isolates with garlic MIC ranging between 3,130 and 12,500 µg/ml. (Ross et al., 2001). On the other hand, almost comparable results have been shown towards multi-drug resistant *E. coli,* where a mean garlic MIC of 20,800±6,100 was observed (Iwalokun et al., 2004). In addition, Abubakar (2009) reported garlic MIC values of 50,000 µg/ml and 100,000 µg/ml towards a standard laboratory *E. coli* strain and a nosocomial *E. coli* isolate, respectively. The current study also showed an inhibitory effect for garlic on *Enterococcus* spp. with a range of 25,000 to 75,000 µg/ml. Lower values have been reported by Jonkers et al. (1999) and Bokaeian and Bameri (2013); with a range of 4000 to 8000 µg/ml and a range of 4000 to 32,000 µg/ml, respectively. The disparity of antimicrobial potency for garlic observed among studies might be attributed to the geographical variation which affects the intensity and range of antibacterial effects of garlic (Bokaeian and Bameri, 2013). Moreover, the concentrations of the effective components of garlic have been found to vary by its age and method of preparation (Iwalokun et al., 2004; Bokaeian and Bameri, 2013).

An important issue in the treatment of bacterial biofilm infections is the lowered effectiveness of antibiotics (Jakobsen et al., 2012), which arises from multiple mechanisms such as failure of the antibiotic to penetrate the full length of the biofilm

and the reduced bacterial growth rate among some of the cells due to nutrient limitation (Costerton et al.,1999). In the present study, the urinary isolates that were either sensitive or intermediately sensitive to nitrofurantoin when grown as planktonic cells became resistant in their sessile mode of growth. The nitrofurantoin MIC towards biofilm grown *Enterococcus* spp. exceeded 512 µg/ml and its MIC towards biofilm grown *E. coli* isolates rose between two to 32 times compared to their planktonic counterparts. In a study of urinary *E. coli* isolates, Ghanwate (2012) noticed an increase in the nitrofurantoin MIC 17 times from planktonic to sessile growth. It has also been stated that more than 100 times the MIC of antibiotics is required to eradicate cells within a biofilm (Sharma et al., 2009).

An important question in this research was the effect of garlic on already formed biofilms. Garlic MIC exceeded 800,000 µg/ml for the biofilm grown *Enterococcus* spp, and it increased between two to 32 times for the *E. coli* isolates when grown as biofilm. Although their study involved a different genus than ours, Shuford et al. (2005) reported that the *in vitro* activity of fresh garlic extract on a *Candida albicans* biofilm decreased as the biofilm phenotype developed from the early to the mature phase.

The present study set out to determine the effect of combining nitrofurantoin and garlic on the studied urinary isolates. There was a significant decline in the MIC values for each of nitrofurantoin and garlic in combination on the planktonic growth of all isolates compared to their MICs alone. The result of which was mostly synergy or addition, with more synergism in favour of ESBL producing *E. coli* compared to enterococci. These findings further support the idea that garlic combination with antibiotics holds promising effects. Synergism has been observed between garlic and vancomycin against vancomycin resistant enterococci (Jonkers et al., 1999). Garlic has also shown synergy with streptomycin against streptomycin resistant *E. coli* (Palaksha et al., 2010).

However, to the best of our knowledge, previous studies have not addressed the effect of the combination of garlic with antibiotics on already formed biofilms. The MICs for each of nitrofurantoin and garlic towards biofilm grown enterococcal isolates rose to high levels from which we could not expand our studies to test for their effect in combination. However, we were able to test their combined effect on the three biofilm forming ESBL producing *E. coli* isolates. Although their independent MICs on the formed biofilms rose to high levels, surprisingly, the MIC values of each of garlic and nitrofurantoin, when combined, dropped 4 times for garlic and between 2 to 128 times for nitrofurantoin; which resulted in either synergy or addition.

It seems possible that our results concerning combination between garlic and nitrofurantoin might be related to the quorum sensing blocking property of garlic (Bodini et al., 2009), which presumably allowed nitrofurantoin to act more efficiently. Our hypothesis is supported by the work of Rasmussen et al. (2005), who demonstrated almost complete elimination of an established *Pseudomonas aeruginosa* biofilm grown in the presence of garlic extract after being treated by tobramycin, in contrast to an untreated biofilm, grown with garlic, which remained viable and a tobramycin treated biofilm, grown without garlic, where only cells in the top layer were killed.

In conclusion, it appears that combinations may be more useful than individual agents. Our results suggest a possible role for garlic in enhancing the antibacterial activity of nitrofurantoin against planktonic and sessile forms of growth. It is thus recommended that further research would be undertaken by utilizing either crude garlic extract or specifically identified molecules obtained from garlic, to be tested alone and in combination with nitrofurantoin towards more urinary isolates of different genera in planktonic and sessile forms before addressing pharmaceutical companies for future combinatory treatment in UTIs.

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REFERENCES

Abubakar E (2009). Efficacy of crude extracts of garlic (*Allium sativum* Linn.) against nosocomial *Escherichia coli, Staphylococcus aureus, Streptococcus pneumoniae and Pseudomonas aeruginosa.* J. Med. Plant Res. 3: 179-185.

Ankri S, Mirelman D (1999). Antimicrobial properties of allicin from garlic. Microbes Infect. 1: 125-129.

Auer S, Wojna A, Hell M (2010). Oral treatment options for ambulatory patients with urinary tract infections caused by extended-spectrum-beta-lactamase-producing *Escherichia coli.* Antimicrob. Agents Chemother. 54: 4006-4008.

Banerjee SK, Maulik SK (2002). Effect of garlic on cardiovascular disorders: a review. Nutr. J. 1: 4.

Baral R, Timilsina S, Jha P, Bhattarai NR, Poudyal N, Gurung R, Khanal B, Bhattachary SK (2013). Study of antimicrobial susceptibility pattern of Gram positive organisms causing UTI in a tertiary care hospital in eastern region of Nepal. Health Renaiss. 11: 119-124.

Bodini SF, Manfredini S, Epp M, Valentini S, Santori F (2009). Quorum sensing inhibition activity of garlic extract and p-coumaric acid. Lett. Appl. Microbiol. 49(5): 551-555.

Bokaeian M, Bameri Z (2013). *In vitro* antibacterial properties of aqueous garlic extract against multidrug-resistant enterococci. Zahedan J. Res. Med. Sci. 15: 43-49.

Bouza E, San Juan R, Munoz P, Voss A, Kluytmans J (2001). Co-operative group of the European study group on nosocomial infections. A European perspective on nosocomial urinary tract infections II. Report on incidence, clinical characteristics and outcome (ESGNI- 004 study). European study group on nosocomial infections. Clin. Microbiol. Infect. 7: 532–542.

Butt T, Leghari MJ, Mahmood A (2004). *In-vitro* activity of nitrofurantoin in *Enterococcus* urinary tract infection. J. Pak. Med. Assoc. 54: 466-469.

Cai Y, Wang R, Pei F, Liang BB (2007). Antibacterial activity of allicin alone and in combination with β -lactams against *Staphylococcus* spp. and *Pseudomonas aeruginosa*. J. Antibiot. 60: 335–338.

Cetti RJ, Venn S, Woodhouse CR (2009). The risks of long-term ni-

trofurantoin prophylaxis in patients with recurrent urinary tract infection: A recent medico-legal case. BJU Int. 103: 567-569.
Chaudhary U, Aggarwal R (2004). Extended spectrum-lactamases (ESBL) - An emerging threat to clinical therapeutics. Ind. J. Med. Microbiol. 22: 75-80.
Chayakul P, Hortiwakul R, Ingviya N, Chayakul V (2007). Species distribution and antimicrobial susceptibility of enterococci in hospitalized patients in Southern Thailand. J. Infect. Dis. Antimicrob. Agents 24: 49-54.
Christensen GD , Simpson WA , Younger JJ , Baddour LM , Barrett FF, Melton DM , Beachey EH (1985). Adherence of coagulase negative Staphylococci to plastic tissue cultures: a quantitative model for the adherence of Staphylococci to medical devices. J. Clin. Microbiol. 22: 996-1006.
Clinical and Laboratory Standards Institute, CLSI (2013). Performance standards for antimicrobial susceptibility testing; Twenty-third informational supplement. CLSI document M100-S23. CLSI, Wayne, PA.
Comerlato CB, de Resende MC, Caierão J, d'Azevedo PA (2013). Presence of virulence factors in *Enterococcus faecalis* and *Enterococcus faecium* susceptible and resistant to vancomycin. Mem. Inst. Oswaldo Cruz 108: 590-595.
Costerton JW, Stewart PS, Greenberg EP (1999). Bacterial biofilms: A common cause of persistent infections. Science 284: 1318-1322.
Das T, Choudhury AR, Sharma A, Talukder G (1996). Effects of crude garlic extract on mouse chromosomes *in vivo*. Food Chem. Toxicol. 34: 43-47.
Ejrnæs K (2011). Bacterial characteristics of importance for recurrent urinary tract infections caused by *Escherichia coli*. Dan. Med. Bull. 58: B4187.
Flemming K, Klingenberg C, Cavanagh JP, Sletteng M, Stensen W, Svendsen JS, Flægstad T (2009). High *in vitro* antimicrobial activity of synthetic antimicrobial peptidomimetics against staphylococcal biofilms. J. Antimicrob. Chemother. 63: 136 - 145.
Ghanwate NA (2012). Biofilm eradication studies on uropathogenic *E. coli* using ciprofloxacin and nitrofurantoin. Int. J. Pharm. Biomed. Res. 3: 127-131.
Gupta K, Hooton TM, Naber KG, Wullt B , Colgan R, Miller LG, Moran GJ, Nicolle LE, Raz R, Schaeffer AJ, Soper DE (2011). International clinical practice guidelines for the treatment of acute uncomplicated cystitis and pyelonephritis in women: A 2010 update by the Infectious Diseases Society of America and the European Society for Microbiology and Infectious Diseases. Clin. Infect. Dis. 52: e103-e120.
Hancock V, Dahl M, Klemm P (2010). Abolition of biofilm formation in urinary tract *Escherichia coli* and *Klebsiella* isolates by metal interference through competition for fur. Appl. Environ. Microbiol. 76: 3836-3841.
Hassan A, Usman J, Kaleem F, Omair M, Khalid A, Iqbal M (2011). Evaluation of different detection methods of biofilm formation in the clinical isolates. Braz. J. Infect. Dis. 15: 305-311.
Hindi NK (2013). *In vitro* antibacterial activity of aquatic garlic extract, apple vinegar and apple vinegar-garlic extract combination. Am. J. Phytomed. Clin.Therapeutics 1: 42 - 51.
Isenberg HD (2007). Clinical Microbiology Procedures Handbook. Vol. II, Second edition, ASM Press, Washington, DC, USA.
Iwalokun BA, Ogunledun A, Ogbolu DO, Bamiro SB, Jimi-Omojola J (2004). *In vitro* antimicrobial properties of aqueous garlic extract against multidrug-resistant bacteria and *Candida* species from Nigeria. J. Med. Food 7: 327-333.
Jakobsen TH, van Gennip M, Phipps RK, Shanmugham MS, Christensen LD, Alhede M, Skindersoe ME, Rasmussen TB, Friedrich K, Uthe F, Jensen PØ, Moser C, Nielsen KF, Eberl L, Larsen TO, Tanner D, Høiby N, Bjarnsholt T, Givskov M (2012). Ajoene, a sulfur-rich molecule from garlic, inhibits genes controlled by quorum sensing. Antimicrob. Agents Chemother. 56: 2314-2325.
Jonkers D, Sluimer J, Stobberingh E (1999). Effect of garlic on vancomycin-resistant enterococci. Antimicrob. Agents Chemother. 43: 3045.
Karlowsky JA, Lagace´-Wiens PR, Simner PJ, DeCorby MR, Adam HJ, Walkty A, Hoban DJ, Zhanel GG (2011). Antimicrobial resistance in urinary tract pathogens in Canada from 2007 to 2009: Canward surveillance study. Antimicrob. Agents Chemother. 55: 3169–3175.
Karpman E, Kurzrock EA (2004). Adverse reactions of nitrofurantoin, trimethoprim and sulfamethoxazole in children. J. Urol. 172: 448-453.
Kouidhi B, Zmantar T, Mahdouani K, Hentati H, Bakhrouf A (2011). Antibiotic resistance and adhesion properties of oral Enterococci associated to dental caries. BMC Microbiol. 11: 155 - 161.
Lee HJ, Park HS, Kim KH, Kwon TY, Hong SH (2011). Effect of garlic on bacterial biofilm formation on orthodontic wire. Angle Orthod. 81: 895-900.
Marra A, Dib-Hajj F, Lamb L, Kaczmarek F, Shang W, Beckius G, Milici AJ, Medina I, Gootz TD (2007). Enterococcal virulence determinants may be involved in resistance to clinical therapy. Diag. Micr. Infec. Dis. 58: 59–65.
Mukherjee M, Basu S, Mukherjee SK, Majumder M (2013). Multi-drug resistant and extended-spectrum beta-lactamase production in uropathogenic *E. coli* which were isolated from hospitalized patients in Kolkata, India. J. Clin. Diagn. Res. 7: 449-453.
Nataro J, Bopp C, Fields P, Kaper J, Strockbine N (2011). Escherichia, Shigella, and Salmonella. In Versalovic et al. (eds) Manual of Clinical Microbiology. 10th edition. ASM Press, Washington DC, USA. pp. 603 - 606.
Norinder BS , Kves B, Yadav M, Brauner A, Svanborg C (2012). Do *Escherichia coli* strains causing acute cystitis have a distinct virulence repertoire? Microb. Pathog. 52: 10-16.
Novais Â, Vuotto C, Pires J, Montenegro C, Donelli G, Coque TM, Peixe L (2013). Diversity and biofilm-production ability among isolates of *Escherichia coli* phylogroup D belonging to ST69, ST393 and ST405 clonal groups. BMC Microbiol. 13: 144.
Palaksha MN, Ahmed M, Das S (2010). Antibacterial activity of garlic extract on streptomycin-resistant *Staphylococcus aureus* and *Escherichia coli* solely and in synergism with streptomycin. J. Nat. Sci. Biol. Med. 1: 12–15.
Patel JB, Tenover FC, Turnidge JD, Jorgensen JH (2011). Susceptibility Test Methods: Dilution and Disk Diffusion Methods. In Versalovic et al. (eds) Manual of Clinical Microbiology. 10th edition. ASM Press, Washington DC, USA. pp. 1122-1143.
Pillai SK, Moellering RC, Eliopoulos GM (2005). Antimicrobial Combinations. In: Antibiotics in Laboratory Medicine. Victor Lorian MD (ed) Fifth edition, Lippincott Williams, Wilkins, Philadelphia USA. pp. 365-440.
Ponnusamy P, Natarajan V, Sevanan M (2012). *In vitro* biofilm formation by uropathogenic *Escherichia coli* and their antimicrobial susceptibility pattern. Asian Pac. J. Trop. Med. 5: 210-213.
Rafii F, Hansen EB Jr (1998). Isolation of nitrofurantoin-resistant mutants of nitroreductase-producing *Clostridium* spp. strains from the human intestinal tract. Antimicrob. Agents Chemother. 42:1121-1126.
Rasmussen TB, Bjarnsholt T, Skindersoe ME, Hentzer M, Kristoffersen P, Köte M., Nielsen J, Eberl L, Givskov M (2005). Screening for quorum-sensing inhibitors (QSI) by use of a novel genetic system, the QSI selector. J Bacteriol. 187: 1799–1814.
Ross ZM, O'Gara EA, Hill DJ, Sleightholme HV, Maslin DJ (2001). Antimicrobial properties of garlic oil against human enteric bacteria: Evaluation of methodologies and comparisons with garlic oil sulfides and garlic powder. Appl. Environ. Microbiol. 67: 475–480.
Sandoe JA, Witherden IR, Cove JH, Heritage J, Wilcox MH (2003). Correlation between enterococcal biofilm formation *in vitro* and medical-device-related infection potential *in vivo.* J. Med. Microbiol. 52: 547–550.
Seno Y, Kariyama R, Mitsuhata R, Monden K, Kumon H (2005). Clinical implications of biofilm formation By *Enterococcus faecalis* in the urinary tract. Acta Med. Okayama 59: 79-87.
Sharafadinzadeh N, Moghtaderi A, Alavi-Naini R (2008). Nitrofurantoin-induced peripheral neuropathy: A lesson to be re-learnt. Neurol. India 56: 94-96.
Sharma M, Aparna, Yadav S, Chaudhary U (2009). Biofilm production in uropathogenic Escherichia coli. Indian J. Pathol. Microbiol. 52:294.
Shuford JA, Steckelberg JM, Patel R (2005). Effects of fresh garlic extract on *Candida albicans* biofilms. Antimicrob. Agents Chemother. 49: 473.
Subramanian P, Shanmugam N, Sivaraman U, Kumar S, Selvaraj S (2012). Antiobiotic resistance pattern of biofilm-forming uropathogens isolated from catheterized patients in Pondicherry, India. Australas. Med. J. 5: 344-348.
Tasbakan MI, Pullukcu H, Sipahi OR, Yamazhan T, Ulusoy S (2012).

Nitrofurantoin in the treatment of extended-spectrum β-lactamase-producing *Escherichia coli*-related lower urinary tract infection. Int. J. Antimicrob. Agents 40: 554-556.
Teixeira L, Da Gloria M, Carvalho S, Shewmaker P, Facklam R (2011). Enterococcus. In Versalovic et al. (eds) Manual of Clinical Microbiology. 10th edition. ASM Press, Washington DC, USA. pp. 350 - 364.
Upadhyaya GP, Lingadevaru UB, Lingegowda RK (2011). Comparative study among clinical and commensal isolates of *Enterococcus faecalis* for presence of *esp* gene and biofilm production. J. Infect. Dev. Ctries. 5: 365-369.
Wu Y, Qu W, Geng D, Liang JY, Luo YL (2012). Phenols and flavonoids from the aerial part of Euphorbia hirta. Chin. J. Nat. Med. 10: 40-42.

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Full Length Research Paper

Distribution of vancomycin resistant enterococci and their resistance patterns determined by surveillance

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In order to identify the status of vancomycin resistant enterococi (VRE) carrier in our hospital, periodical rectal swab cultures were obtained from the patients hospitalized in the Pediatrics Unit, Neurosurgery Intensive Care Unit and Reanimation Unit. VRE strains isolated were examined for type identification, antibiotic sensitivity, High Level Gentamicin Resistance (HLGR), High Level Streptomycin Resistance (HLSR), beta-lactamase production and genotypic resistance patterns. Rectal swab cultures were obtained from 250 patients and 38 of these (15%) were VRE positive. 28 (73.6%) of the enterococci were *Enterococcus faecium*, 6 (15.8%) were *Enterococcus casseliflavus*, 3 (7.9%) were *Enterococcus gallinarum* and 1 (2.7%) was *Enterococcus faecalis*. 24 strains were identified to have Van A resistance pattern. None of the strains have beta-lactamase. HLGR was identified at a rate of 92% and HLSR at a rate of 95%. In addition to glycopeptide resistance, VRE strains had high levels of Ampicillin, Penicillin, Erythromycin, Rifampicin, Chloramphenicol and Nitrofurantoin resistance. Quinolon resistance was found to be at moderate level (34%, 45%), while Tetracycline (29%), Fosfomycine (6%), Dalfopristin-quinupristin (3%) and Linezolid (0%) were found to be the most effective antimicrobials.

Key words: Enterococcus, rectal colonization, vancomycin resistance.

INTRODUCTION

Vancomycin resistance in Enterococci was first described in 1988 and then resistant strains became widespread worldwide (Uttley et al., 1998). Asymptomatic VRE colonization can easily lead to infections. VRE infections are important causes of mortality and morbidity and their treatment is expensive (Milestone et al., 2010). Early identification of VRE colonization is important for the control of VRE infections. The most important VRE reservoirs are those patients who carry VRE in their gastrointestinal systems (Robert et al., 2005). If surveillance cultures are not performed on patients having high risk, asymptomatic carriership can easily be missed. Standard culture methods and molecular techniques like PCR are important for the identification of VRE colonization and prevention of outbreaks.

The first VRE strain in our hospital was isolated in December 2004 from the urine of a patient hospitalized in our Pediatrics Unit. Considering the possibility of an outbreak, Infection Control Committee was alarmed. By putting into place an active surveillance program, rectal

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Abbreviations: VRE, Vancomycin resistant enterococi; **HLGR,** High Level Gentamycin Resistance; **HLSR,** High Level Streptomycin Resistance.

Table 1. Amplification of Van A, Van B, Van C genes by PCR: Primer series for Multiplex PCR method.

Gene	Position	Primers(5'-3')	Product
Van A	130	CAT GAA TAG AAT AAA AGT TGC AAT A	1030
	1136	CCC CTT TAA CGC TAA TAC GAT CAA	
Van B	138	GTG ACA AAC CGG AGG CGA GGA	433
	570	CCG CCA TCC TCC TGC AAA AAA	
Van C	126	GAA AGA CAA CAG GAA GAC CGC	796
	921	ATC GCA TCA CAA GCA CCA ATC	

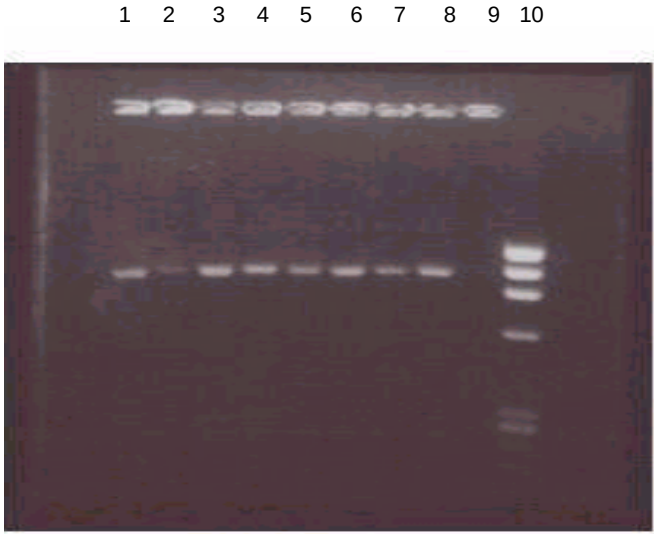

Figure 1. Amplification products which was produced by multiplex PCR reaction on electrophoresis gel. Column 1-7, Van A (1030 bp) positive isolates; Column 8, positive control; Column 9, negative control; Column 10, Marker (ϕx174 Hae III).

swab cultures were obtained from patients under high risk. As anticipated, these patients had a considerably high rate of rectal carriership (15%) in one year surveillance cultures. For the VRE strains isolated, type level identification, antibiotic sensitivity, high level amino-glycoside resistance (HLAR), high level streptomycin resistance (HLSR), beta-lactamase production and genotypic resistance patterns were investigated.

MATERIALS AND METHODS

During the 8-month period from December 2004 to July 2005, VRE carriers' status was investigated by obtaining rectal swab cultures from the patients in the Pediatrics Unit, Neurosurgery Intensive Care Unit, Reanimation Unit and Hematology Units within the first 72 h of their hospitalization. This was followed by a weekly swab during the course of their hospitalization. Rectal swab samples were obtained with sterile swabs. The materials were transferred onto VRE agar medium (OXOID) containing 6 µ/ml Vancomycin and 1 µg/ml Meropenem; type level identification was performed with classical methods and Rapid ID 32 STREP kit (Biomerieux) on the gray-black colonies that were formed after 24-48 h. The identification of antibiotic sensitivities and HLAR was based on CLSI recommendations (CLSI, 2005) and was performed with agar dilution method and ATB Enterocci 5 (Biomerieux) kit. Antibiogram sensitivities to Fosfomycine and Linezolid were studied with disc diffusion method. The confirmation of the strains identified as Vancomycin resistant was performed by E test (AB Biodisk Sweden) with the identification of MIC values as well as identification of resistance genes by Multiplex PCR (Table 1).

A single bacterial colony was taken overnight and grown on a blood agar plate which was suspended in a 100 µl of a PCR mixture containing distillated water (65.5 µl), 10 mM PCR Buffer (10x), 50 mM $MgCl_2$, 0.2 mM dNTP (dATP, dCTP, dGTP,dTTP), 0.5 µm primer and 25 U Taq DNA polymerase. Thermocycler conditions were as follows: an initial cycle of 94°C for 5 min lysis and denaturation followed by 30 cycles; at 94°C for 30 s denaturation, at 58 C for 30 seconds primer binding, at 72°C for 30 s elongation of primer; at the end of 30 cycles at 72°C for 10 min: all tubes were put in the thermocycler and at the end of 30 cycles, PCR products were analyzed by electrophoresis on a 1.5 % agarose gel with etidium bromide and on a UV transilluminator (Figure 1). Beta-lactamase activity was analyzed with Nitrocefin discs (OXOID).

Table 2. Resistance rates to antibiotics.

Antibiotic	Resistant bacteria	Resistance rates (%)
Penicillin	38	100
Ampicillin	38	100
Erythromycin	38	100
Rifampycin	38	100
Vancomycin	38	100
Teicoplanin	38	100
Nitrofurantoin	29	76
Chloramphenicol	29	76
Ciprofloxacin	17	45
Levofloxacin	3	34
Tetracycline	11	29
Fosfomycin (n:35)	2	6
Dalfopristin-Quinupristin	1	3
Linezolid (n:35)	0	0
HLGR	35	92
HLSR	36	95

RESULTS

From December 2004 to July 2005, rectal swab samples were obtained from 250 patients. 38 (15%) of these were VRE positive and repetitive strains were excluded from the study. Of these patients, 23 (60.5%) were hospitalized in the Infant Unit of the Pediatrics Department, 4 were in the Reanimation Unit, 9 were (23.7%) in the Neurosurgery Intensive Care Unit and 2 were leukemia patients in the Internal Medicine Department.

Of the 38 VRE strains studied, 73.6% (28) were *E. faecium*, 15.8% (6) were *E. casseliflavus*, 7.9% (3) were *E. gallinarum*, and 2.7% (1) were *E. faecalis*. All the isolated VRE strains were found to be resistant to Teicoplanin, Ampicillin, Penicillin, Erythromycin and Rifampicin. Antibiotic resistance rates are shown in Table 2. 35 isolate (92%) had HLGR and 36 (95%) isolates had HLSR. Of the 38 colonized patients, none developed infections.

Twenty four (24) strains could be studied for resistance genes with PCR which resulted in the identification of Van A type resistance pattern. All isolates exhibited resistance to vancomycin, teicoplanin, ampicillin, penicillin, erythromycin and rifampicin, which were all *E. faecium.* Clonal relationship among the isolates was not investigated. But all the strains were *E. faecium*, Van A pattern and had same antibiotic resistance. None of the investigated strains had beta-lactamase positivity.

DISCUSSION

First, VRE strains were reported in the United Kingdom in 1988 and this was immediately followed by reports from France and USA; but the spread was very fast (Uttley et al., 1989). The first VRE strain in our country was reported in 1998 from Akdeniz University and this was followed by other case reports (Kocagöz et al., 1999). In these cases, most of the strains studied for phenotype were reported as *E. faecium* with Van A resistance pattern (Başustaoğlu et al., 2000; Çetinkaya et al., 2004; Gündeş et al., 2002; Yiş et al., 2011; Ergani et al., 2008). In the year 2000, in a multicenter study done in Europe on 4208 enterococcus isolates, it was determined that prevalence of Van A and Van B phenotype for Turkey was about 1-2% (Schouten et al., 2000). In the studies reported abroad, the isolated strains also had Van A resistance pattern (Song et al., 2009). Of the 38 strains isolated in our hospital, resistance genes were investigated in 24. And similar to the findings of the previous studies, all of the samples were found to be *E.faecium* with Van A resistance pattern.

Different studies report different rates of VRE colonization and infections. Harris et al. (2004) covered 42 surgical intensive care units in their study; of the 1362 cases they studied, they reported VRE colonization in 136 (10%). In the studies reported from the USA, rectal VRE colonization rates in the intensive care units are reported as 6-20% (van den Braak et al., 2000; Song et al., 2009; Kim et al., 2012). In the studies done in Pediatric ICUs, rectal carriership rates are reported as 2-5% (Milestone et al., 2010; Gray et al., 2000). In our study, we had 15% rectal colonization rate, which is similar to high colonization rates of VRE.

Of the enterococci strains, *E. faecalis* is the most commonly identified strain as the cause of infections. *E. faecium*, on the other hand, is more resistant to antimicrobials compared to *E. faecalis* and can spread easily clonally, so it is accepted as a more important pathogen (Arias et al., 2008). In recently performed studies, *E. faecium* is being isolated more. *E. faecalis* to *E.faecium* rate was 3/1 in 2002 while it was reported as 1.2/1 in 2006

(Lester et al., 2008). In our study, for enterococci colonization, *E. faecium* had a higher rate than *E. faecalis*. Of the 38 VRE strains isolated, 28 (73%) were defined as *E. faecium*. Song et al. (2009) identified 33 *E. faecium* and 1 *E. faecalis* in their study.

The recommended treatment for VRE infections is the combination of an aminoglycoside with Penicillin, Ampicillin or Amoxicillin. However, Vancomycin resistant strains are usually resistant to other antibiotics as well (Harris et al., 2004). The widespread existence of HLAR is a significant problem in the treatment of enterococcal infections. HLAR eliminates the synergistic effects of the beta-lactame+aminoglycoside combination used for treatment and results in treatment failures (Agarwal et al., 2009). In our study, all the VRE strains were found to be resistant to Penicillin, Ampicillin, Erythromycin and Rifampicin. Furthermore, HLGR was identified as 92% and HLSR as 95%. Resistance to Chloramphenicol was 76%; to Nitrofurantoin, 75%; to Ciprofloxacin, 45%; and to Levofloxacin, 34%. High level aminoglycoside resistance is 28-44 % in the South American hospitals (Panesso et al., 2010). Based on EARRS data, it was 15.4 -50 % in Europe. 26 European countries reported 6950 isolates of which 2484 had high level resistant to aminoglycosides (EARS-Net, 2009). High level aminoglycoside resistance is 30-60% in the USA and is reported to be most frequent in *E. faecium* (Taşova, 2009; EASAC reports, 2007). In a study by Çetinkaya et al. (2004) on 49 strains, all the VRE strains that were isolated within the scope of the surveillance program at Hacettepe University Hospital were identified to have Teicoplanin and Ampicillin resistance, as well as HLGR and HLSR. Nitrofurantoin resistance was identified as 95.7%, Rifampicin resistance as 78.7%, Tetracycline resistance as 55.3% and Chloramphenicol resistance as 17.7%. The rate of Tetracycline resistance was higher than that we identified. Similar to our study, beta-lactamase positivity was not identified. Decrease of PBP or production of beta-lactamase by enterococci is responsible for the resistance to beta-lactame antibiotics. That is why beta-lactamase activity should be analyzed in enterococci (Robert et al., 2005). In a study by Gordon et al. (1992) on 705 strains, beta-lactamase positivity of enterococci was reported as 1.6%. In the study by Agarwal et al. (2009), only one out of 86 enterococci was identified to have beta-lactamase positivity.

All the strains in our study were found to be sensitive to Linezolid. In a study in Canada, from January 2010 to June 2012, of 2829 enterococcal isolates tested, 12 *E. faecium* were found to be resistant to linezolid (Patel et. al., 2013). According to LEADER Surveillance Program 2011, activity of linezolid on enterococci is 99.7% (Flamm et al., 2013). In our study, resistance rate to Fosfomycine was identified as 6%. However, the use of Fosfomycine is only recommended for *E. faecalis* strains (Robert et al., 2005). Dalfopristin-Quinupristin is also effective for *E. faecium* strains. In a study in the USA performed by E test, 87% of the strains were found to be sensitive (Lamb et al., 1999). In our study, *E. faecium* constituted 73.6% of the VRE strains and the sensitivity rate was found to be high.

In conclusion, in the VRE strains examined in our study, in addition to glycopeptide resistance, there were high levels of HLAR, Penicillin, Ampicillin, Erythromycin, Rifampicin, Chloramphenicol and Nitrofurantoin resistance. Quinolon resistance was of moderate degree (34 and 45%). Tetracycline (29%), Phosphomycine (6%), Dalfopristin-Quinupristin (3%) and Linezolid (0%) were the most effective antibiotics.

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REFERENCES

Agarwal J, Kalyan R, Singh M (2009). High Level Aminoglycoside Resistance and Beta-Lactamase Production in Enterococci of a Tertiary Care Hospital in India Jpn. J. Infect. Dis. 62: 158-9.

Arias CA, Murray B (2008). Emergence and management of drug-resistant enterococcal infections. Expert Rev. Anti Infect. Ther. 6(5):637-55.

Başustaoğlu A, Özyurt M, Beyan C (2000). Glycopeptide resistant Enterococcus isolated from blood cultures. Flora 5:142-7.

Çetinkaya Y, Zarakolu P, Altun B, Kaya G, Yıldırım A, Ünal S (2004). Epidemiological properties of Vancomycin Resistant Enterococci which were isolated from Hacettepe University. XXXI. Congress of Turkish Microbiology, congress book, p. 263.

CLSI (2005). Performans Standards for Antimicrbial Susceptibility Testing; Eleventh Informational Supplement. CLSI document M 100-S 17; Ankara, Society of Turkish Microbiology.

Ergani ÖA, Naas T, Baysan B (2008). Nosocomial outbreak of Vancomycin Resistant Enterococcus faecium in a paediatric unit at a Turkish university hospital. J. Antimicrob. Chemother. 61(5):1033-9.

Flamm R, Mendes R, Ross J, Sader H, Jones R (2013). Linezolid Surveillance Results for the United States: LEADER Surveillance Program 2011. Antimicrob. Agents Chemother. 57(2): 1077-1081.

Gordon S, Swenson JM, Hill BC, Pigott NE, Facklam RR, Cooksey RC (1992). Antimicrobial susceptibility patterns of common and species of enterococci causing infections in the United States. Enterococcal study group. J. Clin. Microbiol. 30(9):2373-2378.

Gray JW, George RH (2000). Experience of Vancomycin resistant enterococci in a Children's Hospital. J. Hosp. Infect. 45:11-18.

Gündeş S, Wilke A, Ateş B (2002). Results of point prevalance study following first Vancomycin resistant Enterococcus isolation in Hospital of Kocaeli University. J. KLIMIK 5: 3: 78-81.

Harris AD, Nemoy L, Johnson JA (2004). Co-carriage rates of VRE and extended spectrum beta-lactamase producing bacteria among a cohort of ICU patients:implications for an active surveillance program. Infect. Control Hosp. Epidemiol. 25(2):105-8.

Kim YJ, Kim S, Kim YR, Lee JY, Park YJ, Kowg MW (2012). Risk factors for VRE and mortality in colonized patients on intensive care unit admission. AJIC Dec, 40(10):1018-1019.

Kocagöz S (1999). First identification of Vancomycin resistant enterococcus in Turkey. J. ANKEM 13: 5-6.

Lamb HM, Figgitt DP, Fouolds D (1999). Quinopristine- Dalfopristine: A review of its use in the management of serious gram positive infections. Drugs 58:1061-97.

Lester CH, Sandvang D, Olsen S (2008). Emergence of ampicillin resistant Enterococcus faecium in Danish hospitals. J. Antimicrob. Chemother. 62(6): 1203-1206.

Milestone A, Maragakis L, Conroll K, Perl T (2010). Targeted Surveillance to Identify Children Colonized with Vancomycin Resistant Enterococcus in the Pediatric Intensive Care Unit. Inf. Contr. Hosp. Epidemiol. 31(1): 94-97.

Panesso D, Reyes J, Rincon S, Diaz L, Gallaway -Pena J, Zurita J (2010). Molecular epidemiology of Vancomycin Resistant Enterococcus faecium: a prospective multicenter study in South American hospitals. J. Clin. Microbiol. 48:1562-9.

Patel S, Memari N, Shakinas D, Toye B, Jamieson FB, Farell D (2013). Linezolid resistance in Enterococcus faecium isolated in Ontari, Canada. Diagn Micr. Inf. Dis. 77(4):350-53.

Robert C, Moellering JR (2005). Enterococcus species, Streptococcus bovis and Leuconostoc species.In Mandell GL, Bennett JE, Dolin R (eds). Principles and Practice of Infectious Diseases. Sixth edition, New York, Churchill Livingstone. 2411-2421.

Song JY; Cheong HJ, Jom Y, Choi WS, Noh YJ, Heo JY, Kim WJ (2009). Vancomycin -Resistant Enterococcus colonization before admission to the intensive care unit: A clinicoepidemiologic analysis. Am. J. Infect Control 37:734-40.

Taşova Y (2009). How Have the Etiologic Agents been Changed? What is in our Hand now? J. ANKEM 23: 25-36.

Uttley AC, Collins CH, Naidoo J, George RC (1998). Vancomycin resistant enterococci. Lancet. 1:57-8.

Van den Braak, Ott A, van Belkum A, Kluytmans JA, Koleman JG (2000). Prevalance and determinants of fecal colonization with VRE in hospitalized patients in the Netherlands. Infect. Control Hosp. Epidemiol. 21:520-4.

Yiş R, Aslan S, Cıtak C, Değirmenci S (2011). Evaluation of VRE colonization at Gaziantep Children's Hospital, Turkey. Microbiol. Bull. (4):646-54.

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African Journal of Microbiology Research

Full Length Research Paper

Isolation and classification of fungi associated with spoilage of post-harvest mango (*Mangifera indica* L.) in Saudi Arabia

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A survey was conducted between May and July to assess the extent of loss in mangoes at wholesale and consumer levels caused by fungal spoilage during post-harvest. Mango fruits were purchased from different markets in Saudi Arabia, and the degree of losses due to fungal spoilage was assessed at the different levels of marketing. Fungal spoilage was found to be the highest at the consumer level and least at the wholesale level. *Aspergillus flavus* rot, *Aspergillus niger* rot, and *Penicillium* spp. rot were the commonest diseases affecting the mango fruits.

Key words: *Aspergillus*, *Penicillium*, rot and spoilage.

INTRODUCTION

Fruits are widely cultivated in large quantities in different parts of the world. One of the factors compromising the economic value of fruits is the reduction in their shelf-life due to post-harvest disease. The recommended quantity of fruits to be consumed by a normal healthy adult is 230 g/day, while the current per capita consumption of fruits is reported to be less than 160 g/day (Veeraragavathatham et al., 1996). Mango originated from India and Southeast Asia; it is one of the most important fruits cultivated in tropical countries. Mango cultivation has now extended to several other parts of the world including Africa, the Americas and the Caribbean region. Mango is one of the most popular fruits in the tropical region and is increasingly being consumed in the developed countries (Diedhiou et al., 2007). Post- harvest losses in fruits can be attributed to several factors, the most important of which is post-harvest disease. The post-harvest losses of fresh mango fruits are reported to be 25 - 40% in India and 69% in Pakistan; and microbial decay accounts for 17.0 - 26.9% of the total post-harvest losses in Asian countries (Prabakar et al., 2005). The percentage loss of fruit over the marketable period has been reported to be the highest for mango (Mandal and Dasgupta, 1981). The potential of mango as a commercial crop is markedly limited because of its high perishability, which results in considerable wastage (Mootoo, 1992). In addition, mango fruits are susceptible to post- harvest diseases, extremes of temperature, and physical injury (Crucifix and Pilgrim, 2001). Mango fruit diseases of major concern to producers are anthracnose caused by *Colletotrichum gloeosporioides* (Penz.) and stem-end rot caused by *Botryosphaeria parva* (Swart et al., 2002). Several factors affect mango production with post-harvest losses being one of the major constraints (Theodosy and Elde, 2011). In Saudi Arabia, mango is imported from different tropical countries, sold at different levels of marketing and consumed as a fresh fruit.

The objectives of this study were to assess the extent and nature of fungal spoilage in fresh mangoes imported to Saudi Arabia and to identify the main causative factors.

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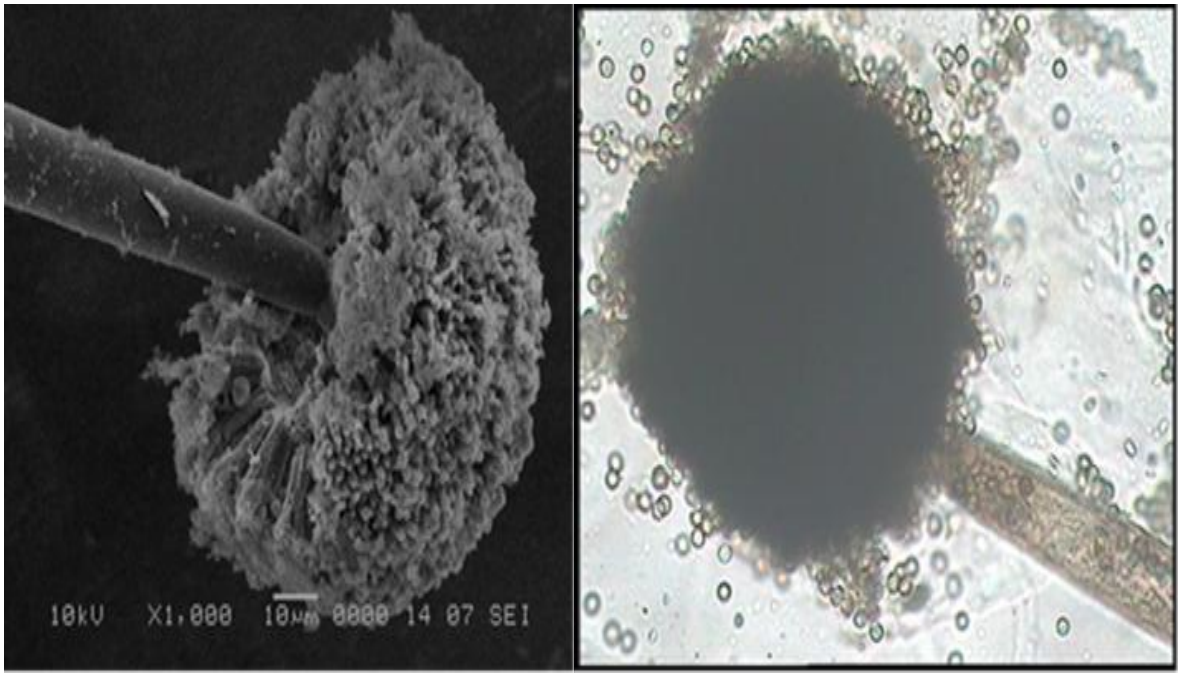

Figure 1. Microscopic and electronmicroscopic images of *Aspergillus niger*.

MATERIALS AND METHODS

A survey was conducted to assess the extent of loss in mango fruits caused by fungal rots during post-harvest. 100 samples were purchased in all, from different markets in Saudi Arabia. The samples were sorted to identify infected mangoes, which were then transferred into sterile polythene bags, labeled, and assessed in the laboratory. The loss due to fungal rots was assessed at weekly intervals for three months (May to July 2012). 85% out of 100 samples were infected. The prevalence was calculated using the following formula:

$$\text{Prevalence \%} = \frac{\text{Number of locations with diseased mangoes}}{\text{Total number of locations}} \times 100$$

During the survey, infected mango fruits from different fruits market of Saudi Arabia were collected in sterile polythene bags, labeled and taken to the laboratory. Fungal species were isolated from each spoiled fruit, incubated at 28°C for 10 days, and identified using Potato Dextrose Agar (PDA). Pure colonies of fungal isolates were classified according to the conventional guidelines of fungus identification (Ellis, 1971; Samson and Varga, 2007). The pathogenicity of the isolated fungal species was confirmed by inoculating them in 250 ml Erlenmeyer flasks containing 5% fresh uninfected mango peels under aseptic conditions, to induce rotting. The inoculated flasks were incubated at 28°C in a rotary incubator shaker with shaking at 150 rpm for five days.

RESULTS AND DISCUSSION

Overall, the prevalence of mango spoilage due to disease was determined to be 85%. The fungal species isolated from the infected mangoes were *A. flavus*, *A. niger* and *Penicillium* spp. (Figures 1 and 2). Mangoes affected with *A. niger* rot showed brown circular spots with depressions, which then enlarged into darker lesions. Mangoes infected with *Penicillium* spp. rot showed a large number of bluish green spores (Tables 1 and 2). *Aspergillus* spp. infection was noted widely among all the examined spoiled mangoes. The point of entry of the pathogenic fungi was believed to be injured and weakened areas of the mango flesh. *A. niger* var. Tieghem (IMI No. 29005) was isolated from a spoiled ripe mango.

The severity of *A. flavus* infection was tested at different temperatures by incubating mango fruits inoculated with the fungus, and the severity of *A. flavus* rot was found to be highest at 35°C and 100% relative humidity (RH) (Gadgile and Chavan, 2010). After harvest, mangoes are susceptible to infection by several fungi such as *A. flavus*, *A. niger*, and *Penicillium* spp. Artificial infection studies have shown that fruits are susceptible to infection at all stages of ripeness (Palejwala et al., 1987).

Analysis of the spoilage at different time points between May and July revealed that the possibility of fungal infection was highest in the month of July. Additionally, our data on spoilage at different stages of marketing revealed that the spoilage was highest at consumer level and least at wholesale level. In post-harvest condition, mangoes get infected by several fungal diseases like *A. flavus*, *A. niger* and *Penicillium* spp.. *A. flavus* was investigated by incubating inoculated mango fruits at different temperatures, and at 35°C and 100%

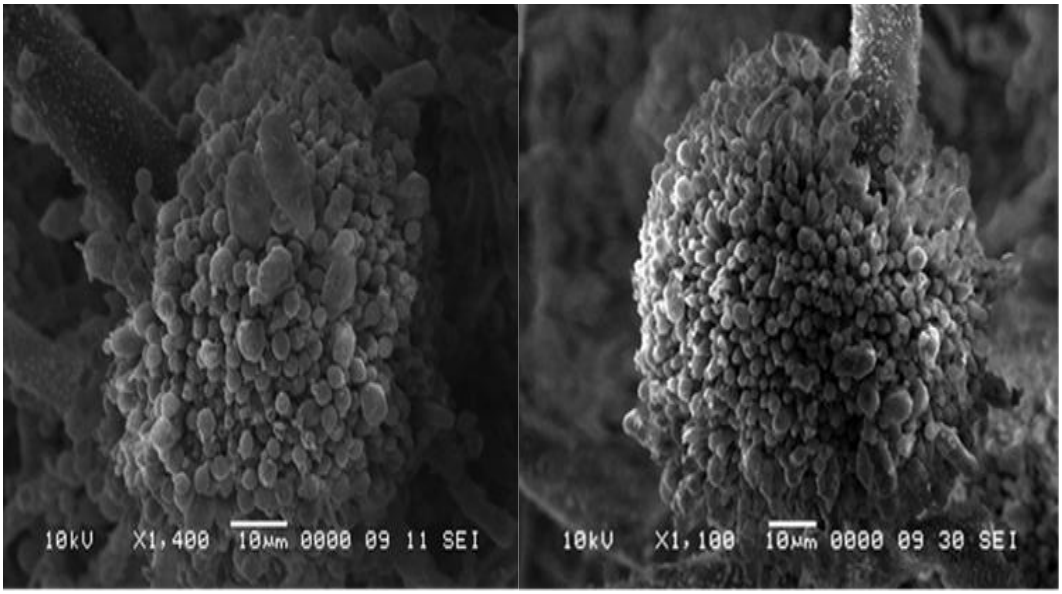

Figure 2. Electronmicroscopic images of*Aspergillus flavus* .

Table 1. Symptoms of post-harvest diseases in mango, as recorded between May and July, 2012.

Disease	Spoilage fungus	Symptoms
Aspergillus niger rot	*Aspergillus niger*	Brown circular spots, that enlarge to form darker lesions
Aspergillus flavus rot	*Aspergillus flavus*	Powdery yellow green spores
Blue- mould rot	*Penicillium* spp.	Bluish- green spores

Table 2. Prevalence of post-harvest diseases in mango, as recorded between May and July, 2012.

Disease	Spoilage fungus	Prevalence (%)
Aspergillus niger rot	*Aspergillus niger*	100
Aspergillus flavus rot	*Aspergillus flavus*	100
Blue- mould rot	*Penicillium* spp.	100

R.H *A. flavus* rot severity was maximum (Gadgile and Chavan, 2010).

Conclusion

Mangoes are highly perishable fruits and very prone to fungal infection. It is clear that fungal spoilage like *A. flavus* rot, A*. niger* rot, and Penicillium spp. were the commonest fungal diseases affecting mangoes, especially, in developing countries where storage and handling techniques are primitive. Our findings reveal that fungal spoilage of mango was highest in the month of July at consumer level and least at the wholesale level.

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REFERENCES

Crucifix D, Pilgrim R (2001). Quality and quality standard. In: Robins G. Julie mango in the Eastern Caribbean. CTA, Postbus 380, 6700 AJ Wageningeon, the Netherlands pp. 7-10.

Diedhiou PM, Mbaye N, Drame A, Samb PI (2007). Alteration of postharvest diseases of mango Mangiferaindica, through production practices and climatic factors. Afr. J. Biotechnol. 6(9):1087-1094.

Ellis MB (1971) *Dematiaceoushyphomycetes*, Cab international, Wallingford, UK.

Gadgile DP, Chavan AM (2010) Impact of temperature and relative humidity on development of *Aspergillusflavus* rot of mango fruit. Sci.

Technol. 3:48-9.
Mandal NC, Dasgupta MK (1981). Post-harvest diseases of perishables in West Bengal II. Losses. Ann. Agric. Res. 273-85.
Mootoo A (1992). Efforts to improve the post harvest technology of mangoes and heliconias. In: The Caribbean Agricultural Research and development institute (CARDI) CTA seminar proceeding S. post-harvest management of tropical fruits and ornamentals in Caribbean region. pp. 167-175.
Palejwala VA, Patki CK, Bhatt, SV, Modi VV (1987). Post-harvest spoilage of mangoes by Aspergillusniger. Int. J. Food Microbiol. 5:111-116.
Prabakar K, Raguchander T, Parthiban VK, Muthulakshmi P, Prakasam V (2005). Post harvest fungal spoilage in mango at different levels marketing. Madras Agric. J. 92(1-3):42-48.
Samson RA, Varga J (2007). *Aspergillus* systematics in the genomicera. CBS Fungal Biodiversity Centre, Utrecht. p. 206.
Swart SH, Serfontein JJ, Kallinowski J (2002). Chemical control of postharvest diseases of mango-the effect of prochloraz, thiabendazole and fludioxonil on soft brown rot, stem-end rot and anthracnose S.A. Mango Growers' Assoc. Res. J. Year Book. 22:55-62.
Theodosy JM, Elde SK (2011). Assessment and management of post harvest losses of fresh mango under small-scale business in Morogoro, Tanzania. J. Anim. Plant Sci. 11(1):1358-1363.
Veeraragavathatham D, Jawaharlal M, Jeeva S, Rabindran R (1996). Scientific Fruit culture,Suri Associates, Coimbatore. p. 227.

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Full Length Research Paper

Polyphasic identification of isolates of *Chromobacterium* sp. obtained from flooded soil

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The bacterium *Chromobacterium violaceum* had its genome sequenced by the Genome Consortium in 2002, revealing a number of genes with potential for use in pharmacological and industrial biotechnology. However, only a few studies have been conducted to establish the molecular characterization of the different isolates of *C. violaceum*. Thus, the present study aimed to identify the isolates of *Chromobacterium* through their fatty acid profile and partial sequencing of the 16S rDNA gene. The results show that, unlike previously studied isolates, the profiles of UCP1035, UCP1489 and UCP1466 contained the saturated fatty acid palmitoleic (C16: 1). Corroborating data from the 16S rDNA phylogeny revealed that although most isolates from *Chromobacterium* were grouped as cluster 1 isolates, UCP1035 and UCP1466 formed monophyletic branches. The isolate UCP1489 and isolates from *Chromobacterium piscinae* were grouped in cluster 2. The data obtained from each *Chromobacterium* species in the tree showed that only the UCP1462, UCP1469 and UCP1470 isolates exhibited 100% identity with the species *C. violaceum* ATCC12472 and *Chromobacterium pseudoviolaceum* LMG3953T. These data shed light on the genetic diversity of bacteria whose genes may have biological functions but have not yet been characterized for their biotechnological potential.

Key words: *Chromobacterium violaceum*, *Chromobacterium pseudoviolaceum*, fingerprinting, fatty acids, 16S rDNA, genetic diversity.

INTRODUCTION

The genus *Chromobacterium* belongs to the family Neisseriaceae, and the *Chromobacterium violaceum* species was first proposed by Bergonzini (Boisbaudran, 1882). One of the most notable characteristics of this bacterium is the production of a violet pigment called violacein, when grown under aerobic conditions (Rettori, 1996). *C. violaceum* is a Gram-negative saprophyte found in soil and water samples of the tropical and subtropical regions of several continents (Durán and Menck, 2001). In Brazil, it is mostly found on the banks of the Rio Negro in the Amazon. The Brazilian National Genome Project Consortium executed the sequencing and analysis of the genome of *C. violaceum* (Brgene, 2003). Brazil is included among the countries that exhibit an interest in the DNA of this organism, as it is responsible for the production of a variety of secondary metabolites with biotechnological potential (Carraro et al., 2004).

So far, six species of the genus *Chromobacterium* have

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Table 1. List of *Chromobacterium* isolates, origin and sequence of 16S rDNA.

Number collection	Origin	Substratum	Size bp	GenBank accession
UCP-1461	Amazon	Flooded soil	986	EU880909.1
UCP-1462	Amazon	Flooded soil	987	EU880910.1
UCP-1463	Amazon	Flooded soil	988	EU880911.1
UCP-1464	Amazon	Flooded soil	988	EU880912.1
UCP-1465	Amazon	Flooded soil	965	EU880913.1
UCP-1466	Amazon	Flooded soil	985	EU880914.1
UCP-1467	Amazon	Flooded soil	980	KJ158054
UCP-1468	Amazon	Flooded soil	989	EU880915.1
UCP-1469	Amazon	Flooded soil	978	EU880916.1
UCP-1470	Amazon	Flooded soil	988	EU880917.1
UCP-1471	Amazon	Flooded soil	987	EU880918.1
UCP-1489	Pernambuco	Creek in Paulista	987	EU880919.1
UCP-1035	Ceará	Lagoon Almécegas	987	EU880920.1

been identified, namely *C. violaceum* (Boisbaudran, 1882), *Chromobacterium subtsugae* (Martin et al., 2007), *Chromobacterium piscinae, Chromobacterium pseudoviolaceum* (Kampfer et al., 2009), *Chromobacterium haemolyticum* (Han et al., 2008) and *Chromobacterium aquaticum* (Young et al., 2008). However, the last two species do not exhibit the typical pigment violacein. Of the other distinguishing features, the 16S rRNA genes among the five new types of *C. violaceum* ATCC12472 species display an average sequence similarity ranging from 96.0 to 99.8% between species (Martin et al., 2007; Young et al., 2008).

The application of biochemical and molecular techniques in environmental microbiology has facilitated the identification and characterization of new bacterial species. A tool used in determining taxonomic bacterial species is the analysis of fatty acid methyl ester (FAME) (Osterhout et al., 1991). According to Stead et al. (1992), bacterial species can be easily identified using cellular fatty acid profiles, and this method has been found to possess a high level of accuracy.

The sequencing and analysis of the 16S rDNA (Harmsen and Karch, 2004) has been used successfully in diversity studies, and for the identification and classification of various bacteria. The coupling of molecular analysis methods with systematic bacterial and phylogenetic analysis software has identified the relationships between bacterial groups (Turenne et al., 2001; Cohan, 2002). Thus, the present study aimed to identify the isolates of *Chromobacterium* through their fatty acid profile and by partial sequencing of the 16S rDNA gene.

MATERIALS AND METHODS

Microorganisms and culture conditions

The standard strain of *C. violaceum* ATCC12472 and the other strains that were investigated are listed in Table 1. These isolates were obtained from the collection of the Department of Environmental Sciences at the Catholic University of Pernambuco. The isolates were grown in 250 ml Erlenmeyer flasks containing 50 mL of the LB liquid culture medium (Sambrook and Russel, 2006). The flasks were incubated at 30°C with constant shaking at 150 rpm for 48 h. At the end of the incubation period, the cells were centrifuged at 1,700 x *g* for 10 min at 4°C. The cell mass was subjected to freeze-drying for the subsequent extraction of fatty acids**.**

Extraction and methylation of fatty acids

The fatty acids were converted to methyl esters according to the method of Dunlap and Perry (1967). The lyophilized biomass (100 mg) was placed into a screw cap tube, mixed with 2 ml of a solution of boron trifluoride in 14% methanol and 2 mL of benzene and then incubated overnight at 60°C. Following incubation, 2 ml of distilled water was added to the mixture and the tubes were agitated in a vortex for 5 min. The mixture was centrifuged at 1700 xg for 10 min at 40°C. Following centrifugation, the benzene was removed and evaporated with nitrogen, while the fatty acid methyl esters were resuspended in n-hexane and analyzed by gas chromatography (GC).

Gas chromatography

The analysis were performed on a gas chromatograph model Varian CP-3380 coupled to CP-8200 autosampler, capillary column CP SIL 8CB (30 m x 0.25 mm), using helium as the carrier gas. The temperature of the injector and detector (FID) was maintained at 250°C, the temperature "oven" was initially set at 130°C, subsequently increased to 170°C at the rate of 1 to 3°C/min and was finally maintained isothermally at 180°C for 10 min. The fatty acids were identified by comparing the retention times of the peaks of the samples with the standards and the relative amounts of methyl ester accomplished by the composition of cellular fatty acids CFA were calculated by integration of the peak areas. A range of fatty acids standards was obtained from Sigma.

DNA extraction

The *Chromobacterium* isolates were grown on solid LB medium for

48 h at 30°C. The colonies were transferred to test tubes containing 5 ml of liquid LB medium for 48 h at a temperature of 30°C, kept under constant agitation at 150 rpm and then subjected to DNA extraction. The DNA of the isolates was extracted according to the method described by Weisburg (1991). The material obtained was quantified on a 1.0% agarose gel run at 80 V for 30 min in TBE buffer (Sambrook and Russel, 2006) stained with SYBR gold and visualized and photographed with the 1D Image Analysis Software System (Kodak Digital Science) under UV light.

DNA amplification of the 16S rRNA gene

The 16S rRNA gene present in the isolates was amplified by PCR using the universal primers fD1 and rD1 (Weisburg et al., 1991). The reaction containing 200 mM deoxynucleotidyl, 5 pmoles of each primer, 2 mM $MgCl_2$, 10X PCR buffer, 1.25 U Taq DNA polymerase (Invitrogen) and 40 ng of the template DNA was performed in the Thermal Cycler Gene Amp ® PCR System 9700 (Applied Biosystems), with an initial cycle of denaturation at 94°C for 5 min, then 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 45 s and extension at 72°C for 1 min and 30 s, followed by a final extension at 72°C for 7 min. The amplicons were separated by electrophoresis at 100 V on a 1.0% agarose gel in 0.5 X TBE buffer (Sambrook and Russel, 2006) and stained with Sybr Gold (Invitrogen); fragments with the expected size were visualized under UV light and photographed with the 1D Image Analysis Software system (Kodak Digital Science) under UV light. The 1 Kb Plus DNA Ladder (Invitrogen) was used as a marker.

Sequencing of the 16S rDNA gene fragment

The amplified products were purified with the PCR Kit "GFX TM PCR DNA" (Invitrogen) to remove residual nucleotides, primers and enzyme, thus permitting a sequencing quality reaction. The purified material was sequenced on the MegaBace sequencer (GE Healthcare) using the same primers as those used in the amplification reaction. The sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) program to query the nucleotide similarity to sequences in the GenBank database.

Phylogenetic analysis

The 16S rDNA sequences were aligned and compared using the program "ClustalX V.4.0." (Larkin et al., 2007). Furthermore, this comparison was conducted with the GenBank sequences, with the aim of combining the bacteria in their respective groups. For distance matrix and phylogenetic tree construction, we used the Tamura-Nei neighbor-joining algorithm processed by the program Molecular Evolution Genetics Analysis MEGA version 5.0 (Tamura et al., 2007). Gene sequences from *C. violaceum* (NR07422), *C. subtsugae* (JX500187), *C. haemolyticum* (JX500185), *C. aquaticum* (KC201360), *C. piscinae* (JN566136), *C. pseudoviolaceum* (AJ871128), obtained from the GenBank database, were included.

RESULTS

The results of the GC-Gas chromatography analysis identified the presence of fatty acids: lauric acid C12: 0, myristic acid C14: 0, palmitic acid C16: 0, Palmitoleic acid C16: 1 and linolelaidic acid C18: 2. The profiles of the two principal fatty acids that characterize the isolates of *Chromobacterium* were C16: 0 (palmitic acid) and C16:1 (palmitoleic). For the fatty acid C12: 0, the lowest amount was produced in the isolate UCP1035 (4.2%) while the highest amount was produced in the isolate UCP1463 (8.5%); however, this fatty acid was not detected in the isolate UCP1471. In all the bacterial species, only a small percentage of fatty acids are freely expressed and can be easily isolated by extracting the cells with the appropriate organic solvent. For the fatty acid C14:0 expressed in isolates of *Chromobacterium*, the lowest amount was retrieved in the isolate UCP1462 (5.3%), while the highest amount was in UCP1468 (13.4%). In contrast, the highest concentration of the fatty acid C18: 2 was detected in the isolates UCP1462, UCP1465, UCP1466 and UCP1471 at 24.0, 22.0, 20.0 and 22.0%, respectively (Figures 1 and 2). A reduction in the concentration of C18: 2 was observed in the UCP1461 and UCP1467 isolates, which had relatively low concentrations (3.0%) of the fatty acid; this result confirms the C18: 2 content (2.0%) observed in the standard strain ATCC12472.

A comparison of the 16S rDNA sequences of isolates in our study with sequences from the GenBank (NCBI) confirmed the entire genus *Chromobacterium*. The phylogenetic tree (Figure 3) of these sequences constructed using the Tamura-Nei model for nucleotides and pairwise deletion with 1000 bootstrap replicates showed that all the isolates (UCP1461, UCP1462, UCP1463, UCP1464, UCP1468, UCP1470 and UCP1471) belong to the same group. The 16S rRNA gene of the *Chromobacterium* isolates showed fragments of sizes ranging from 848-942 bp. There was a high degree of sequence identity in the 16S rDNA gene from all isolates of the *Chromobacterium* species (Table 2); the sequences of 12 of the isolates exhibited 98% identity with the sequence of the 16S rDNA of *C. violaceum* ATCC 12472. The highest identity between the isolates was among the species *C. violaceum* and *C. pseudoviolaceum*, which showed a 100% identity between the isolates (UCP 1462, UCP1463, UCP 1469 and 1470), while the isolate UCP1489 showed an identity with *C. subtsugae* (100%). The tree based on these sequences confirmed the close phylogenetic relationship of the isolates of *Chromobacterium* (UCP1461, UCP1462, UCP1463, UCP1464, UCP1468, UCP1470 and UCP1471) with *C. violaceum* and *C. pseudoviolaceum* (Figure 2). Furthermore, the resulting tree revealed two clusters: cluster 1 grouped all the isolates from the Amazon region and the Ceara and was further divided into four sub-clusters. The first sub-cluster included seven isolates of *Chromobacterium*, sub-cluster 2 included isolated UCP1465 and the species *C. violaceum* and *C. pseudoviolaceum*, and finally the sub-clusters 3 and 4 were monophyletic with the branch isolates UCP1035 and UCP1466, respectively. Cluster 2 had 2 sub-clusters: the first sub-cluster with isolated UCP1489 with the species *C. subtsugae* and *C. piscinae* confirmed that the isolate did not group together based on their origin and the second sub-cluster with the

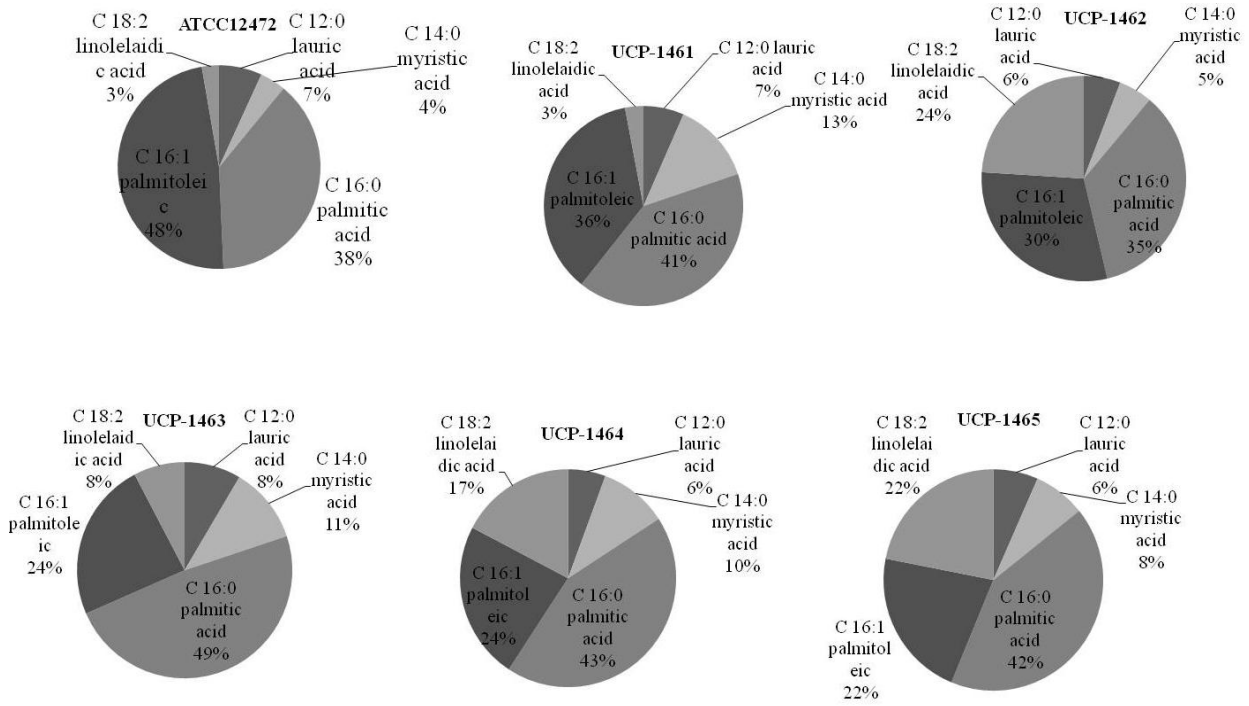

Figure 1. Fatty acid composition of type strain of *C. violaceum* ATCC12472 and isolated UCP1461, UCP1462, UCP1463, UCP1464 and UCP1465.

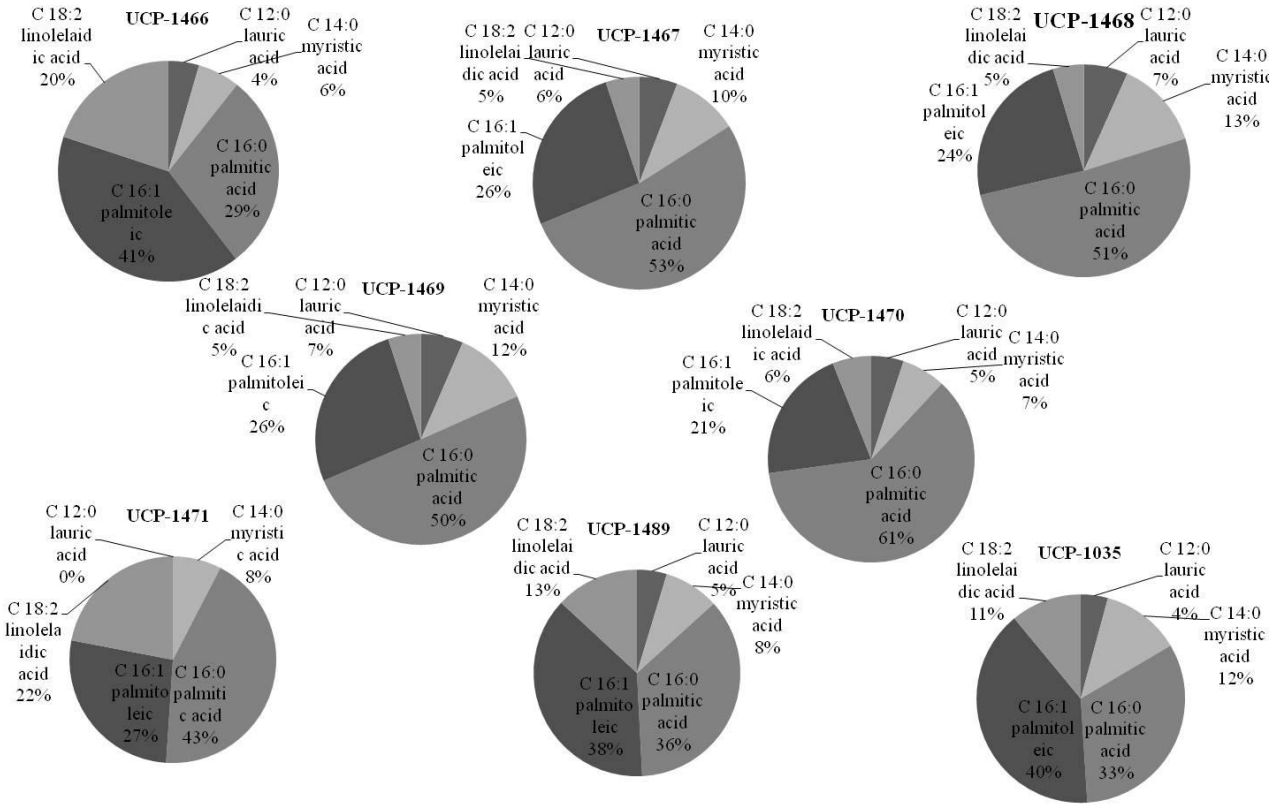

Figure 2. Fatty acid composition of isolates UCP1466, UCP1467, UCP1468, UCP1469, UCP1470, UCP1471 (all from Amazon), UCP1489 (Pernambuco State) and UCP1035 (Ceará State).

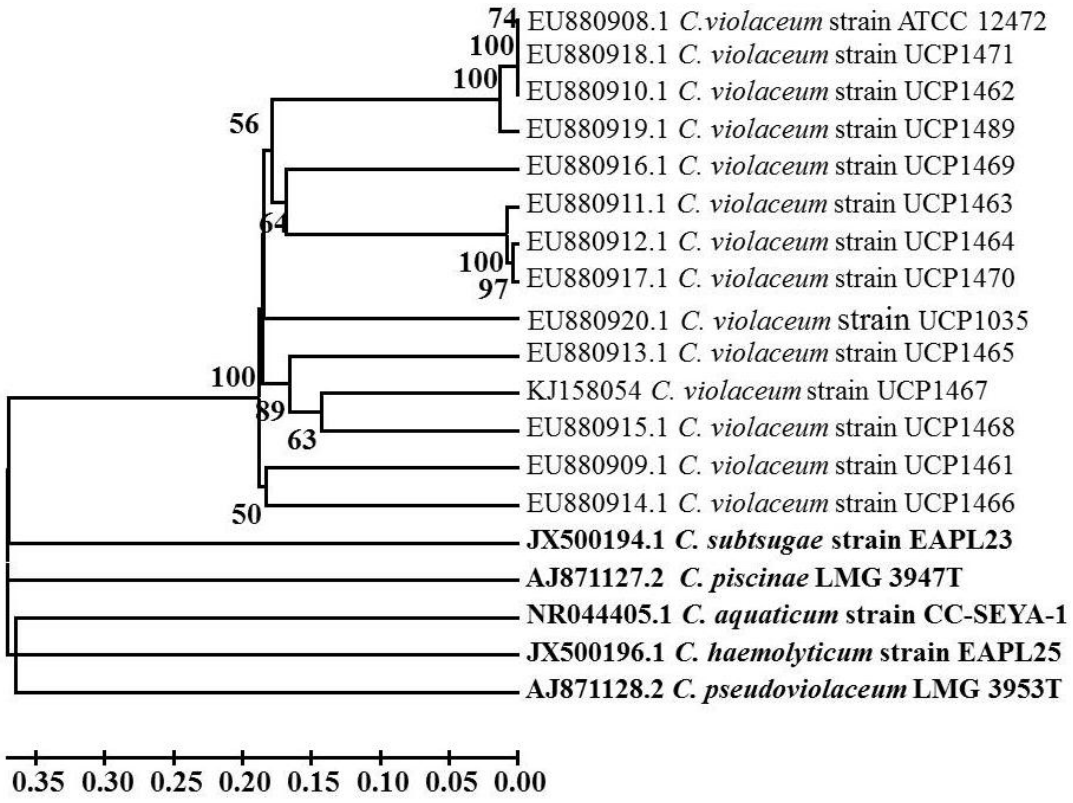

Figure 3. Phylogenetic tree based on sequences of 16S rDNA genes of all isolates of *Chromobacterium violaceum*: UCP1461, UCP1462, UCP1463, UCP1464, UCP1465, UCP1466, UCP1467, UCP1468, UCP1469, UCP1470, UCP1471 (Amazon State), UCP1489 (Pernambuco State) and UCP1035 (Ceará State) obtained by the Neighbor-Joining method with bootstrap 1000 and Tamura-Nei model (Mega5).

species *C. aquaticum* and *C. heamolyticum*. The results obtained with the 16S rRNA can be corroborated by the similarities observed with sequences deposited in the database, which can be assessed following this type of analysis. The data obtained in each tree species, separately with *Chromobacterium*, only isolated UCP1462, UCP1469 and UCP1470 and showed 100% identity with the species *C. violaceum* ATCC12472 and *C. pseudoviolaceum* LMG3953T (Table 2 and Figures 3, 4 and 5).

DISCUSSION

The highest levels of fatty acids were found in the standard strain ATCC12472 were C16: 0 (palmitic acid) and C16: 1 (palmitoleic acid). Additionally, the fatty acid composition that we identified corroborates previous findings by other groups in bacteria (Oliver and Colwell, 1973; Kamimura et al., 1992). For most of the isolates that were analyzed, the highest observed concentration among the saturated fatty acids was that of palmitic acid (C16: 0), except for the UCP1466, UCP1489 and UCP1035 which exhibited higher amounts of palmitoleic acid (C16: 1) at 40.7, 38.0 and 40.0%, respectively. Delong and Yayanos (1985), working with *Vibrio*, reported the presence of fatty acids C16: 1 and C18: 1. The major problem involved in the study of bacterial fatty acids is identifying an extraction procedure in which all the fatty acids that act on cells are well characterized because the number of cells required to obtain an optimum extraction varies with the bacterial species (Pacheco, 2009)

The similarity between the isolated strain and the UCP1469 standard *C. violaceum* ATCC 12472 was 100%. Moreover, the isolates collected in the Amazonas grouped separately from those collected in Ceará and Pernambuco States. The complete sequence of the 16S rDNA can stratify the position of the bacteria at the genus level and, in some cases, at the species level as well (Garrity and Holt, 2001). Thus, the 16S rDNA must be used, preferably in studies of taxonomy and phylogeny, with an effort to deposit a large number of these sequences related to other ribosomal genes. Sequencing of the 16S rDNA gene has been frequently used as a molecular marker, making this sequence useful in the genomic fingerprinting of bacteria (Lima-Bittencourt et al., 2011) Corroborating the results of the authors cited in the

Table 2. Maximum identity of 16S rDNA sequences among isolates and *Chromobacterium* species *C. violaceum*, *C. pseudoviolaceum*; *C. subsugae*; *C. piscinae*; *C. haemolyticum*, *C. aquaticum* from The National Center for Biotechnology Information (NCBI).

Description of the accesses of the isolates of *Chromobacterium violaceum*	*C. violaceum* ATCC12472	*C. pseudoviolaceum* LMG3953T	*C. subtsugae* EAPL16	*C. piscinae* LS8	*C. haemolyticum* EAPL14	*C. aquaticum* HME8586
gi|213391470|gb|EU880909.1| *C. violaceum* strain UCP1461	99%	99%	98%	98%	96%	96%
gi|213391471|gb|EU880910.1| *C. violaceum* strain UCP1462	**100%**	**100%**	98%	98%	96%	95%
gi|213391472|gb|EU880911.1| *C. violaceum* strain UCP1463	100%	100%	98%	98%	96%	95%
gi|213391473|gb|EU880912.1| *C. violaceum* strain UCP1464	99%	99%	98%	98%	96%	95%
gi|213391474|gb|EU880913.1| *C. violaceum* strain UCP1465	100%	100%	98%	98%	96%	95%
gi|213391475|gb|EU880914.1| *C. violaceum* strain UCP1466	98%	98%	99%	99%	97%	97%
gi|213391476|gb|EU880915.1| *C. violaceum* strain UCP1468	99%	99%	98%	98%	96%	95%
gi|213391477|gb|EU880916.1| *C. violaceum* strain UCP1469	**100%**	**100%**	98%	97%	95%	95%
gi|213391478|gb|EU880917.1| *C. violaceum* strain UCP1470	**100%**	**100%**	98%	98%	96%	95%
gi|213391479|gb|EU880918.1| *C. violaceum* strain UCP1471	99%	99%	98%	98%	96%	96%
gi|213391480|gb|EU880919.1| *C. violaceum* strain UCP1489	98%	98%	**100%**	99%	97%	98%
gi|213391481|gb|EU880920.1| *C. violaceum* strain UCP1035	98%	98%	99%	99%	96%	96%

last paragraph, we found that in isolated *Chromobacterium* Cerrado (Brazil) a 97.8% similarity existed with the sequence of the 16S rDNA gene of *C. violaceum* ATCC 12472. Stackebrandt and Goebel (1994) proposed a similarity in the sequences of ≥97% for delimiting bacterial species. However, the 16S rRNA is considered a neutral marker, used to distinguish bacteria to the species level according to their evolutionary history of common ancestry, although with some limitations (Oren, 2004).

The characterization of bacteria can be effectively performed by identifying and characterizing the sequence information obtained from the 16S rDNA of several samples of *Chromobacterium* sp., as previously accomplished by Lima-Bittencourt et al. (2011).

A similar result was observed by Hungria et al. (2005) on analyzing the 16S rDNA sequences of *C. violaceum* to identify the genetic diversity among isolates collected from UFAM in Rio Negro - Brazilian Amazon; the study identified two new groups of species of *C. violaceum* as has been previously described. We know that microorganism

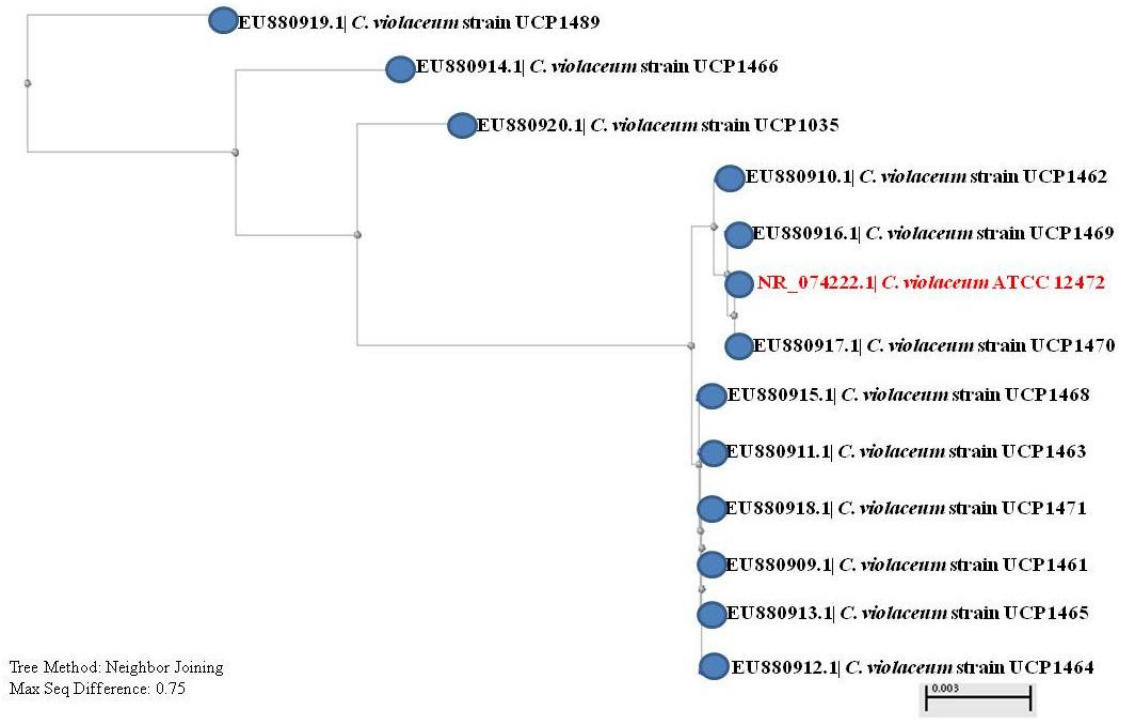

Figure 4. Identity maximum of 16S rDNA sequences among isolates NR_074222.1 *C. violaceum* ATCC 12472 with the isolates studied in this work using the method of constructing phylogenetic Neighbor Joining with maximum parsimony analysis 0.75

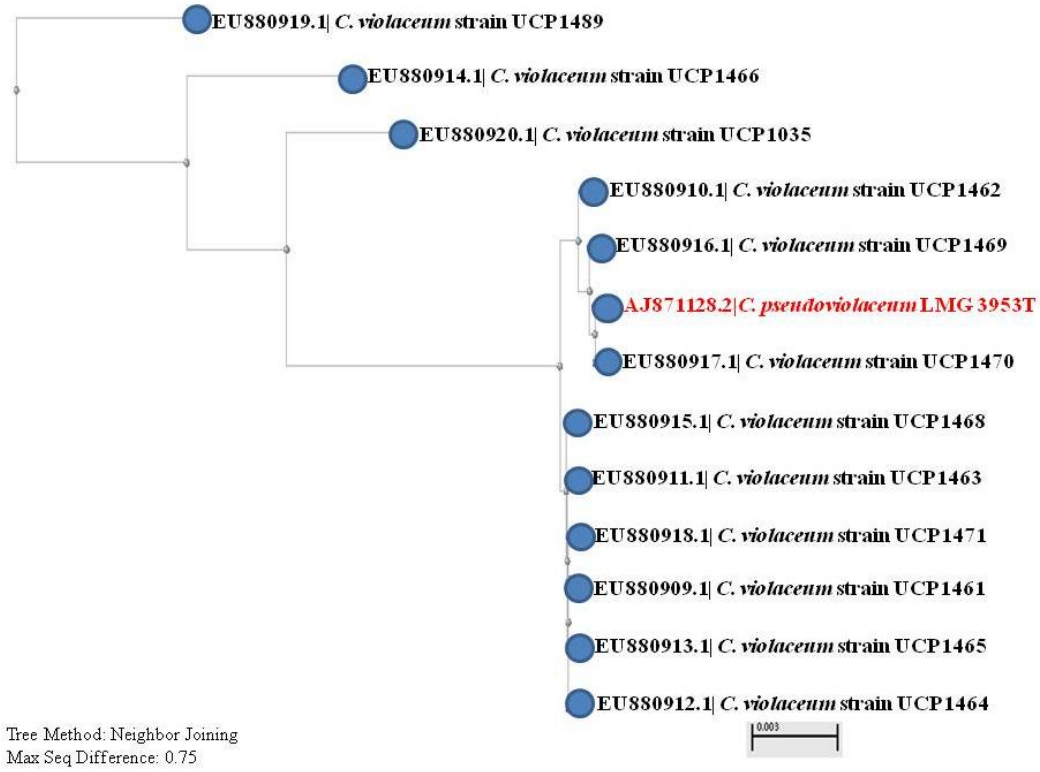

Figure 5. Maximum identity of 16S rDNA sequences among isolates AJ871128.2 *C. pseudoviolaceum* LMG 3953T with isolates studied in this work using the method of constructing phylogenetic Neighbor Joining with maximum parsimony analysis 0.75.

play an essential role in stabilizing the ecosystem through recycling and degradation of pollutant compounds. The *C. violaceum* has a great biotechnological potential and new techniques for bioprospecting and biodiversity can be an important source of genetic resources in the pharmaceutical, cosmetic and food industry; additionally, these new species of *C. violaceum* can significantly contribute to the discovery of new compounds and metabolites. The saturated fatty acid profile (specifically palmitoleic (C16: 1) exhibited by the isolates UCP1035, UCP1489 and UCP1466 was different from that of the other isolates studied. The confirmation of the phylogeny of the 16S rDNA gene revealed that most isolates of *Chromobacterium* were grouped in cluster 1; however, UCP1035 and UCP1466 formed monophyletic branches. Grouped in cluster 2 were UCP1489 and *C. piscinae* alone. The isolates UCP1462, UCP1469 and UCP1470 shared 100% identity with the strains *C. violaceum* and *C. pseudoviolaceum*.

Fatty acids have been used as a tool to study the polyphasic, which is a chemotaxonomic method easier to analyze a large number of isolates in a short period of time. Concomitantly, we also used the sequencing technique of 16S rDNA gene which is more complex and aimed to give more credibility to the obtained results.

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REFERENCES

Boisbaudran LD (1882). Matiére colorante se formant dans la colle de farine. CR. Acad. Sci. 94:562.

Brgene (2003). The complete genome sequence of *Chromobacterium violaceum* reveals remarkable and exploitable bacterial adaptability. Proc. Natl. Acad. Sci. U.S.A. 100(20):11660-11665.

Carraro DM, Camargo AA, Salim ACM, Gonzaga L, Costa GC, Vasconcelos AT, Simpson AJ (2004). Closure of rRNA related gaps in the *Chromobacterium violaceum* genome with the PCR-assisted contig extension (PACE) protocol. Genet. Mol. Res. 3(1):53-63.

Cohan FM (2002). What are bacterial species? Annu. Rev. Microbiol. 56: 457-487.

Delong EF, Yayanos AA (1985). Adaptation of the membrane lipids of a deep-sea bacterium to changes in hydrostatic pressure. Science 228 (4703):1101-1103.

Dunlapk R, Perry J (1967). Effect of substrate on the fatty acid composition of hydrocarbon substituted utilizing microorganisms. J. Bacteriol. 94(6):1919-1923.

Durán N, Menck CF (2001). *Chromobacterium violaceum*: a review of pharmacological and industrial perspectives. Crit. Rev. Microbiol. 27(3):201-222.

Garrity GM, Holt JG (2001). The road map to the Manual. In: Garrity GM, Boone DR, Castenholz RW (eds) Bergey's manual of systematic bacteriology. 2nd ed. New York: The Williams & Wilkins/Springer-Verlag. pp.119-154.

Han XY, Han FS, Segal J (2008). *Chromobacterium haemolyticum* sp. nov., a strongly haemolytic species. Int. J. Syst. Evol. Microbiol. 58(6):1398-1403.

Harmsen D, Karch H (2004). How close is close enough? Not the tree of live but a living tree is of major importance for 16S rDNA based microbial species identification. ASM News 70:19-24.

Hungria M, Astolfi-Filho S, Chueire LMO, Nicolas MF, Santos EB, Bulbol MR, Souza-Filho A, Nogueira AE, Germano MG, Vasconcelos AT (2005). Genetic characterization of *Chromobacterium* isolates from black water environments in the Brazilian Amazon. Lett. Appl. Microbiol. 41(1):17-23.

Kamimura K, Fuse H, Takimura O, Yamaoka Y, Ohwada K, Hashimoto J (1992). Pressure-Induced Alteration in fatty Acid Composition of Barotolerant Deep-Sea Bacterium. J. Oceanog. 48(1):93-104.

Kampfer P, Busse HJ, Scholz HC (2009).*Chromobacterium piscinae* sp. nov. and *Chromobacterium pseudoviolaceum* sp. nov., from environmental samples. Int. J. Syst. Evol. Microbiol. 59(10):2486-2490.

Larkin MA, Blackshields G, Brown NP, Chenna R., McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higg DG (2007). ClustalW and ClustalX version 2.0. Bioinformatics. 23(21):2947-2948.

Lima-Bittencourt CI, Costa PS, Hollatz C, Raposeiras R, Santos FR, Chartone-Souza E, Nascimento AM (2011). Comparative biogeography of *Chromobacterium* from the neotropics. A. Van. Leeuw. J. Microb. 99(2):355-370.

Martin PA, Gundersen-Rindal D, Blackburn M, Buyer J (2007). *Chromobacterium subtsugae* sp. nov., a *betaproteobacterium* toxic to Colorado potato beetle and other insect pests. Int. J. Syst. Evol. Microbiol. 57(5):993-999.

Oliver JD, Colwell RR (1973). Extractable lipids of gram-negative marine bacteria: fatty-acid composition. Int. J. Syst. Evol. Microbiol. 23(4):442-458.

Oren A (2004). Prokaryote diversity and taxonomy: current status and future challenges. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 359(1444):623-638.

Osterhout GJ, Shull VH, Dick JD (1991). Identification of clinical isolates of Gram-negative non fermentative bacteria by an automated cellular fatty acid identification system. J. Clin. Microbiol. 29(9):1822-1830.

Pacheco FLC (2009). Identificação bacteriana por derivação de ácidos graxos extraídos de células integras, 167p, Dissertação de Mestrado, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo (USP) SP, Brasil.

Rettori D (1996). Produção, extração e purificação da violaceína: um antibiótico produzido por *Chromobacterium violaceum*. 44p. Dissertação de Mestrado, Instituto de Química, Universidade Estadual de Campinas, Campinas, SP, Brasil.

Sambrook J, Russel DW (2006). The condensed protocols from molecular cloning: A laboratory manual. CSH Laboratory Press, Cold Spring Harbor, New York. p.800.

Stackebrandt E, Goebel BM (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. 44(4):846-849.

Stead DE, Sellwood JE, Wilson J, Viney I (1992). Evaluation of a commercial microbial identification system based on fatty acid profiles for rapid, accurate identification of plant pathogenic bacteria. J. Appl. Microbiol. 72(4):315-321.

Tamura K, Dudley J, Nei M, Kumar S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24(8):1596-1599.

Turenne CY, Tschetter L, Wolfe J, Kabani A (2001). Necessity of quality controlled 16S rRNA gene sequence databases: identifying nontuberculous Mycobacterium species. J. Clin. Microbiol. 39(10):3637-3648.

Weisburg WG, Barns SM, Pelletier DA, Lane D (1991). 16S ribosomal DNA amplification for phylogeneic study. J. Bacteriol. 173(2):697-703.

Young CC, Arun AB, Lai WA, Chen WM, Chao JH, Shen FT, Rekha PD, Kämpfer P (2008). *Chromobacterium aquaticum* sp. nov., isolated from spring water samples. Int. J. Syst. Evol. Microbiol. 58(4):877-880.

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Full Length Research Paper

Antimicrobial activities of *Delonix elata* (Bojer ex Hook.) Raf. and *Spathodea campanulata* P. Beauv.

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Methanol and aqueous extracts of *Delonix elata* (Bojer ex Hook.) Raf. (Fabaceae) and *Spathodea campanulata* P. Beauv. (Bignoniaceae) were evaluated for their antimicrobial activities against nine bacterial species: *Bacillus cereus, Staphylococcus aureus, Streptococcus pneumonia, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Salmonella typhi, Proteus vulgaris* and *Shigella flexneri* and two fungal species: *Aspergillus niger* and *Candida albicans*. The susceptibility of the microorganism to the extracts of these plants was compared with each other and with selected antibiotics. All these plants were effective against three or more of the pathogenic microorganisms. This *in vitro* study corroborated the antimicrobial activity of the selected plants used in folklore medicine.

Key words: Medicinal plants, infectious diseases, solvents, disc diffusion methods, microbial pathogens.

INTRODUCTION

Infectious diseases are the world's leading cause of premature deaths, killing almost 50,000 people every day. In recent years, drug resistance to human pathogenic bacteria has been commonly reported from all over the world (Yadev and Khan, 2012). The increasing failures of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents have led to the screening of several medicinal plants for their potential antimicrobial activity. The use of medicinal plants as a source of relief from illness can be traced back to over five million years' written documents of the early civilization in China, India and the near East; it is doubtless an art as old as mankind.

Spathodea campanulata P. Beauv. is a species belonging to the Bignoniaceae family, native of equatorial Africa. It is commonly found and planted in the coffee estates of Munnar, South Tamilnadu and is denoted by the name Malaria Maram (tree). Its flowers and stem bark extracts have shown molluscicidal activity and also employed in diuretic, anti-inflammatory treatments. The leaves are used for kidney diseases, urethra inflamemations and as an antidote against animal poisons. The stem bark preparations are used for enemas, fungus skin diseases, herpes, stomach aches and diarrhea (Adriana et al., 2007; Mendes et al., 1986). Hypoglycemic, anti-HIV and anti-malarial activities were also observed in the stem bark extracts (Niyonzima et al., 1999; Makinde et al., 1988).

Delonix elata L. (Family: Fabaceae) is a deciduous tree of about 2.5-15 m height; it has a spreading, rather rounded crown, crooked poor stem form and drooping branches. The plant is traditionally used for the treatment of abdominal pains, rheumatism and flatulence. The stem bark of this plant is considered as good febrifuge and is much appreciated as an antiperiodic and anti-inflammatory (Abd El and Hegazi, 2011). We report here the results of the antimicrobial properties of extracts from the leaves of *D. elata* and *S. campanulata*.

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MATERIALS AND METHODS

Collection of plant samples

Fresh plant leaves were collected randomly from the gardens and villages of Kovilpatti taluk, Tamilnadu from natural stands. The botanical identity of these plants was confirmed by Dr. V. Sampath Kumar, Scientist- C, Botanical Survey of India (Southern Circle), Coimbatore, Tamilnadu. The voucher specimens are deposited at the Department of Botany, National College (Autonomous), Tiruchirapalli-620 001, Tamilnadu, India.

Preparation of extracts

Aqueous extraction

One hundred gram of dried powder was extracted in distilled water for 6 h at slow heat. Every 2 h it was filtered through eight layers of muslin cloth and centrifuged at 5000 rpm and 5000 g for 15 min. The supernatant was collected. This procedure was repeated twice and after 6 h the supernatant was concentrated to one-fifth of the original volume.

Solvent extraction

100g of dried plant powdered samples was extracted with 200 ml of methanol kept on a rotary shaker for 24 h. Thereafter, it was filtered and centrifuged at 5000 rpm for 15 min. The supernatant was collected and the solvent was evaporated to make the final volume one-fifth of the original volume. It was stored at 4°C in airtight bottles for further studies.

Antimicrobial activity

Microorganisms

Microorganisms were obtained from the Microbial Type Culture Collection centre (MTCC), Chandigarh, India. Amongst eleven microorganisms investigated, nine were bacterial strains viz., *Staphylococcus aureus* MTCC 3160, *Bacillus cereus* MTCC 442, *Streptococcus pneumonia* MTCC 655, *Escherichia coli* MTCC 598, *Pseudomonas aeruginosa* MTCC 42642 *Klebsiella pneumoniae* MTCC 7407, *Salmonella typhi* MTCC 3917, *Proteus vulgaris* MTCC 742 and *Shigella flexneri* MTCC 1457, while the other two were fungal strains viz. *Aspergillus niger* MTCC 2546 and *Candida albicans* MTCC 183. All the bacterial strains were maintained on nutrient while fungi were maintained on potato dextrose agar slants.

Disc diffusion method

Antimicrobial activity was carried out by the disc diffusion method. The antimicrobial assays of aqueous and methanolic extracts were performed according to the method of Bauer et al. (1966). Each plant extract was tested at two different concentrations (100 and 200 µg/ml) to see their inhibitory effects against microbial pathogens. Sterile paper discs (6 mm in diameter) prepared from Whatman No. 1 filter paper was impregnated with drug, containing solution placed on the inoculated agar. The inoculated plates were incubated at 37°C for 24 h. The antibacterial activity was evaluated by measuring the diameter of the inhibition zone for the test microorganisms. The potato dextrose agar plates were inoculated each with fungal culture by point (10 days old cultures) inoculation. The filter paper discs loaded with 100 and 200 µg/ml concentrations of the extracts were placed on test organism-seeded plates. The activity was determined after 72 h of incubation at 28°C. The diameters of the inhibition zones were measured in mm (Taylor et al., 1995). Chloramphenicol and Fluconazole are used as standard antibiotics.

Minimum inhibitory concentration (MIC)

For determination of MIC, 1 ml of broth medium was taken into 10 test tubes for each bacterium. Different concentrations of plant extracts ranging from 0.125 to 8 $\mu g/ml^{-1}$ were incorporated into the broth and the tubes were then inoculated with 0.1 ml of inoculums of respective bacteria (10^5 CFU ml-1) and kept at 37°C for 24 h. The test tube containing the lowest concentration of extract which showed reduction in turbidity when compared with control was regarded as MIC of that extract (Muhamed et al., 2011).

Total activity (TA) determination

Total activity is the volume at which test extract can be diluted with the ability to kill microorganisms. It is calculated by dividing the amount of extract from 1 g plant material by the MIC of the same extract or compound isolated and is expressed in ml/g (Sharma and Kumar, 2009).

AI = Activity Index (IZ developed by extract/IZ developed by standard).

RESULTS AND DISCUSSION

Methanol and aqueous extracts of two different plant species were examined for their antimicrobial activity against the isolated human pathogens and the results are in Tables 1 to 6. Both crude methanol and aqueous extracts of *D. elata* exhibited varying degrees of antimicrobial activities against the test organisms. The 200 µg/ml crude methanol extract showed higher inhibition zone than crude aqueous extract against *S. aureus, S. pneumoniae, B. cereus* and *S. flexneri*, respectively (Table 1). Similarly, 200 µg/ml methanol extract of *S. campanulata* exhibited inhibition zone of 18 mm (AI = 0.666) for *S. pneumoniae* and 14 mm (AI = 0.615) for *S. aureus,* respectively.

The aqueous extract showed highest inhibition zone of 9 mm in (AI = 0.346) for *S. aureus* and 8 mm (AI = 0.296) for *E. coli* (Tables 2 and 6). Antibiotics chloramphenicol and fluconazole have shown greater inhibition zone diameter than that of plant extracts. It had the inhibition zone in the range of 15 to 28 mm. There sre no fungal activities in both plant species. Methanol extract of *D. elata* showed least MIC value that is, 0.125 µg/ml (MBC = 0.250 µg/ml) against S*. aureus* while aqueous extract had moderate activity at 4 µg/ml (MBC = 4 µg/ml) concentration (Table 3). Similarly, the *S. campanulata* methanol extract was found to be highly effective as it has shown very low MIC value (0.125 µg/ml) against *S. aureus* (Table 4). The total activity was highest for methanol extracts of both plants (3.6 and 4.0 ml/g) against *S. aureus* (Tables 5 and 6). Our results support this view as methanol extracts had comparatively more inhibition action

Table 1. Antimicrobial activity of crude extracts of *D. elata* (Bojer ex Hook.) Raf.

Name of the strain	Zone of Inhibition (mm)				
	Methanol (µg/ml)		Aqueous (µg/ml)		Chloramphenicol
	100	200	100	200	
Staphylococcus aureus	10	16	-	10	26
Streptococcus pneumoniae	11	14	-	8	21
Bacillus cereus	-	13	-	-	19
Escherichia coli	-	11	-	-	27
Pseudomonas aeruginosa	8	10	-	-	18
Klbseillae pneumoniea	-	9	-	8	22
Salmonella typhi	10	12	-	-	29
Proteus vulgaris	-	10	-	-	20
Shigella flexneri	-	13	-	-	28
Antifungal activity					**Fluconazole**
Candida albicans	-	-	-	-	15
Aspergillus niger	-	-	-	-	17

Table 2. Antimicrobial activity of crude extracts of *S. campanulata* P. Beauv.

Name of the strain	Zone of Inhibition (mm)				
	Methanol (µg/ml)		Aqueous (µg/ml)		Chloramphenicol
	100	200	100	200	
Staphylococcus aureus	10	14	-	9	26
Streptococcus pneumoniae	10	18	-	-	21
Bacillus cereus	-	-	-	-	19
Escherichia coli	-	12	-	8	27
Pseudomonas aeruginosa	8	11	-	-	18
Klbseillae pneumoniea	-	-	-	-	22
Salmonella typhi	-	10	-	-	29
Proteus vulgaris	-	-	-	-	20
Shigella flexneri	-	12	-	-	28
Antifungal activity					**Fluconazole**
Candida albicans	-	-	-	-	15
Aspergillus niger	-	-	-	-	17

Table 3. The MIC index of methanol and aqueous extracts of *D. elata* (Bojer ex Hook.) Raf.

Name of the strain	Methanol			Aqueous		
	MIC (µg /ml)	MBC (µg /ml)	MIC_{index}	MIC (µg /ml)	MBC (µg /ml)	MIC_{index}
S. aureus	0.125	0.250	2	4	4	1
S. pneumoniae	0.250	0.500	2	-	-	-
B. cereus	1	0.500	0.5	-	-	-
E. coli	1	0.500	0.5	-	-	-
P. aeruginosa	0.500	2	4	-	-	-
K. pneumoniae	2	4	2	-	-	-
S. typhi	1	2	1	-	-	-
P. vulgaris	2	1	0.5	-	-	-
S. flexneri	2	0.500	0.25	-	-	-
C. albicans	-	-	-	-	-	-
A. niger	-	-	-	-	-	-

Table 4. The MIC index of methanol and aqueous extracts of *S. campanulata* P. Beauv.

Name of the strain	Methanol			Aqueous		
	MIC (µg /ml)	MBC (µg /ml)	MIC_{index}	MIC (µg /ml)	MBC (µg /ml)	MIC_{index}
S. aureus	0.125	0.250	2	4	4	1
S. pneumoniae	0.250	0.500	2	-	-	-
B. cereus	-	-	-	-	-	-
E. coli	0.500	0.500	1	-	-	-
P. aeruginosa	1	2	1	-	-	-
K. pneumoniae	-	-	-	-	-	-
S. typhi	2	2	1	-	-	-
P. vulgaris	-	-	-	-	-	-
S. flexneri	0.500	0.500	1	-	-	-
C.albicans	-	-	-	-	-	-
A. niger	-	-	-	-	-	-

Table 5. Activity index and total activity of crude extracts of *D. elata* (Bojer ex Hook.) Raf.

Name of the strain	Methanol			Aqueous		
	Activity index		Total activity (ml/g)	Activity index		Total activity (ml/g)
	100	200		100	200	
S. aureus	0.384	0.615	3.6	-	0.384	0.1
S. pneumoniae	0.523	0.666	1.8	-	0.380	-
B. cereus	-	0.684	0.45	-	-	-
E. coli	-	0.407	0.45	-	-	-
P. aeruginosa	0.444	0.555	0.9	-	-	-
K. pneumoniae	-	0.409	0.225	-	0.363	-
S. typhi	0.344	0.413	0.45	-	-	-
P. vulgaris	-	0.500	0.225	-	-	-
S. flexneri	-	0.464	0.225	-	-	-
C.albicans	-	-	-	-	-	-
A. niger	-	-	-	-	-	-

Table 6. Activity index and Total activity of crude extracts of *Spathodea campanulata* P. Beauv

Name of the strain	Methanol			Aqueous		
	Activity index		Total activity (ml/g)	Activity index		Total activity (ml/g)
	100	200		100	200	
S.aureus	0.384	0.538	4	-	0.346	0.1
St. pneumoniae	0.476	0.857	2	-	-	-
B. cereus	-	-	-	-	-	-
E.coli		0.444	1	-	0.296	-
P.aeruginosa	0.444	0.611	0.5	-	-	-
K. pneumoniae	-	-	-	-	-	-
S. typhi	-	0.344	0.25	-	-	-
P.vulgaris	-	-	-	-	-	-
S. flexneri	-	0.428	1	-	-	-
C.albicans	-	-	-	-	-	-
A.niger	-	-	-	-	-	-

than aqueous extracts (Hugo et al., 2005).

Several workers have reported that many plants possess antimicrobial properties including the parts, that is, flower, bark, stem, leaf, etc (Doss et al., 2009a, b; Sasikumar et al., 2006; Venkataswamy et al., 2010). Recently, a number of plants have been reported for antimicrobial properties across the world. It has been shown that when solvents like ethanol, hexane and methanol are used to extract plants, most of them are able to exhibit inhibitory effect on both gram positive and gram negative bacteria (Palombo and Semple, 2001). In the present study, the methanol extracts of the selected plants also showed zone of inhibition against the isolated human pathogens with varied diameter. In conclusion, the methanol extracts of both plants possess broad spectrum of antibacterial activity against the test bacteria species. The results obtained from this work give high hope for the development of new antibacterial agents.

REFERENCES

Abd El G, Hegazi M (2011). In vitro studies on *Delonix elata* L.-an endangered medicinal plant. World Appl. Sci. J. 14(5):679-686

Adriana P, Jurandir PP, Dalva TF, Noemia KI, Raimundo PF (2007). Iridoid glucose and antifungal phenolic compounds from *Spathodea campanulata* roots, Cien. Agrar, 28:251-256.

Bauer AW, Kirby WMW, Sherris JC, Turck M (1966). Antibiotic susceptibility testing by a standardized single disc method. Am. J. Clin. Pathol. 45:494-496.

Doss A, Dhanabalan R, Sasikumar JM, Palaniswamy M, GeethaV, Ayyappa DM (2009b). Antibacterial activity of twelve medicinal plants from Western Ghats. Pharmacology 1:83-89.

Doss A, Muhammed MH, Dhanabalan R (2009a). Antibacterial activity of tannins from the leaves of *Solanum trilobatum* Linn. Indian J. Sci. Technol. 2(2):41-43.

Hugo JB, Anneleen K, Anders B, William RM, Inga H, Jolanta JL (2005). Antifungal and antibacterial activity of some herbal remedies from Tanzania. J. Ethnopharmacol. 96:461-469.

Makinde JM, Amusan OOG, Adesogan EK (1988). The antimalarial activity of Spathodea campanulata stems bark extract on *Plasmodium berghei berghei* in mice. Plant Med. 54(2):122-25.

Mendes NM, Souza CP, Araujo N, Pereira JP, Katz N (1986). Atividade moluscicida de alguns produtos naturais sobre Biomphalaria Memorias do Instituto Oswaldo Cruz, Rio de Janeiro. 81:87-91.

Muhamed MH, Doss A, Dhanabalan R, Venkataswamy R (2011). Activity of some selected medicinal plants against Bovine Mastitis Pathogens. J. Anim. Vet. Adv. 6:738-741.

Niyonzima G, Laekeman G, Witvrouw M, VanPoel B, Pieters L, Paper D, Clercq E, Franz G, Vlietinck AJ (1999). Hypoglycemic, anticomplement and anti-HIV activities of *Spathodea campanulata* stem bark. Phytomedicine. 6(1):45-49.

Palombo EA, Semple SJ (2001). Antibacterial activity of traditional medicinal plants. J. Ethnopharmacol. 77:151-157.

Sasikumar JM, Pichai Anthoni Doss A, Doss A (2006). Antibacterial activity of *Eupatorium gladulosum*. Fitoterapia. 76(2):240-243.

Sharma B, Kumar P (2009). Extraction and Pharmacological Evaluation of Some Extracts of Tridax procumbens and Capparis deciduas. Int. J. Appl. Res. Nat. Prod. 1(4):5-12.

Taylor RSL, Manandhar NP, Hudson JB, Towers GHN (1995). Screening of selected medicinal plants of Nepal for antimicrobial activities. J. Ethanopharmacol. 546:153-159.

Venkataswamy R, Muhamed Mubarack H, Doss A, Lakshmi Devi J, Sukumar M (2010). Preliminary phytochemical screening and Antimicrobial studies of *Lantana indica* Roxb. Indian J. Pharm. Sci. 72(2):66-68.

Yadav M, Khan KK (2012). Investigations of antibacterial activity of some ethnomedicinal plants against certain pathogenic bacterial strains. Indian J. Sci. 1(2):57-59.

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Full Length Research paper

Production and characterization of alkaline protease from bacteria strains isolated from cotton field

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A number of bacterial strains producing protease enzymes were isolated from the soil of cotton field. Five strains producing high level of extracellular protease with alkaline conditions were selected. Maximum production of 4675 U/ml of protease in 48 h of growth was obtained from strain MW17, while maximum specific activity of 21515U/mg was obtained with strain MW09. pH optimum of 8-10 was observed with different proteases. Protease from strain MW05 showed maximum stability after 24 h of incubation. Strains showed different optimum temperature for protease activity, that is, 30°C (MW08), 40°C (MW05, MW28) and 50°C (MW09, MW17). Mg^{2+}, Fe^{3+} and Zn^{2+} were found to increase the enzyme activity of different strains, while Ca^{2+} decreased the activity. Most of proteases were stable in commercial detergent, thereby, indicating the possible application of these proteases in detergent industry. Selected strain MW09 showing required properties was characterized with respect to physiological and biochemical properties.

Key words: Alkaline protease, cotton, detergent, enzymes.

INTRODUCTION

Enzymes being selective in terms of function and specificity are suitable catalyst as compared to chemical catalysts. Further enzyme controlled reactions can occur in aqueous environment. Enzymes find applications in diagnostic, food and feed, beverages and other biotechnology industry besides application in research and development. The present enzyme industry worldwide is approximately worth $5.8 billion. The demand of enzyme will rise from 6.8% annually to $8.0 billion in 2015 (http://www.freedoniagroup.com/World-Enzymes.html). Among the various enzymes, hydrolytic enzymes have maximum application and commercial values. Enzymes are being isolated from microorganism, plants and animals.

Proteases are group of enzymes that are able to hydrolyze the peptide bonds in proteins and polypeptides and classified as serine protease, cysteine protease, aspartic protease and metallo-protease (Singh et al., 2001). Proteases are used in detergent, food and feed industry, leather industry, photography industry and meat industry. To improve the softness and shining property, proteases are applied on raw silk fibers. They also find applications in oncology, inflammatory conditions, blood rheology control, immune regulation and constitute more that 60% of the total enzyme market. Protease in association with lipase constitute part of lens cleaning solutions for removal of soil particles (Moreira et al., 2002; Najafi et al., 2005; Ray, 2012; Binod et al., 2013; Sathiya, 2013). Wide sources of protease from bacteria, fungi, plant and animal are reported (Shaginian et al., 1990; Wang et al., 2006; Chi et al., 2007; Chu, 2007). Microorganism which is easier to grow under controlled

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conditions is preferred source of enzymes. As micro-organisms can be manipulated to increase production and modify the properties of the enzymes of interest, a lot of work has been reported on microorganisms. Microorganisms have been isolated from various environmental conditions with the aim to have enzyme with special characteristics and applications (Ogawa and Shimizu, 1999; Nigam, 2013). Protease having different pH optimum can be used in variety of applications. Khan (2013) reviewed a number of microbial protease especially alkaline proteases for their industrial applications. The present study involves the isolation, production and characterization of alkaline protease from the micro-organisms with possible applications in industry.

MATERIALS AND METHODS

Isolation of alkaline protease producing microorganisms

Samples of soil from various fields including cotton soil were collected from different parts of Haryana (India). These samples were suitably diluted and plated on the skim milk agar plates containing peptone (1.5%), malt extract (1%), NaCl (0.5%) and skim milk (1%). The plates were incubated at 40°C for 24 h. A clear zone of skim milk hydrolysis indicated protease producing organism. All the strains producing proteases were transferred to skim milk plates having different pH: 8, 9, 10 and 11. Selected strains were processed for future work.

Production and partial purification of alkaline protease

Production of the protease from the selected strains of bacteria was carried out in a medium containing peptone (1.5%), malt extract (1%) and NaCl (0.5%) at temperature of 40°C and 150 rpm. The pH of the medium was adjusted to 10 with 0.1 N NaOH. Samples were taken out at regular interval of 6 h and estimated for optical density, protein and protease activity. After 48 h, cultures were centrifuged at 10,000 rpm, 4°C and supernatant was collected. Supernatants were subjected to ammonium sulphate precipitation (100% saturation) followed by dialysis against glycine-NaOH buffer (0.1 N; pH 10.0). The resulted products were used as partially purified enzyme for characterization of proteases.

Enzyme assay

Protease activity was estimated as per protocol of Singh et al. (2001) with certain modification. To 100 µl of azocasein (1% w/v) in glycine-NaOH buffer (0.1M, pH 10.0), 100 µl of enzyme was added and incubated for 60 min at 40°C (otherwise specified) followed by addition of 1 ml of 10% (w/v) trichloro acetic acid (TCA). Tubes were kept on ice for 15 min and centrifuged at 10,000 rpm for 30 min. One ml of supernatant was mixed with 500 µl of NaOH (1.0 N). Absorbance was measured at 420 nm using appropriate blanks.

One unit (U) enzyme activity was defined as the amount of enzyme required to produce an increase in absorbance equal to 1.0 in 60 min.

Protein assay

Concentration of protein was estimated by the method of Lowry et al. (1951). Routine calibration curve from standard BSA solution (0.02-0.2 mg) was used to measure protein concentration.

Characterization of crude enzyme

Effect of pH on enzyme activity and stability

Activity of the enzyme was measured at different pH values: pH 6.0-8.0 (sodium phosphate buffer); pH 9.0-11.0 (glycine-NaOH buffer, 0.1 N). Mixtures were incubated at 40°C (otherwise specified) and the activity of the enzyme was measured. Enzyme was diluted in different buffers (pH 6.0-11.0) and incubated at 40°C for 24 h and the relative activity was measured as per assay procedure.

Effect of temperature on enzyme activity and stability

The activity of the enzyme was determined by incubating the reaction mixture at different temperature ranging from 30 to 60°C. To determine the enzyme stability with changes in temperature, partial purified enzyme was incubated at different temperatures (37, 45, 50 and 60°C) and relative activities were assayed at standard conditions.

Effect of various metal ions on protease activity

The effects of metal ions e.g. Ca^{2+}, Fe^{3+}, Cu^{2+}, Mg^{2+}, Na^{+} and Zn^{2+} (50 mM) were investigated by adding them into the reaction mixture. Relative protease activity was measured.

Stability of proteases with laundry detergents

The compatibility of partially purified alkaline proteases with local laundry detergents was studied. Detergents used were: Henko (Jyothy Laboratories Ltd); Ariel, Tide (Procter and Gamble, India), Rin detergent, Surf Excel Quickwash, Surf Excel Blue (Hindustan Lever Limited, India). The detergents were diluted in distilled water (1.0%w/v), incubated with protease for 1 h at optimum temperature of each protease. The enzyme activity of a control sample (without detergent) was taken as 100%.

RESULTS AND DISCUSSION

Selection and production of alkaline protease

Thirty nine (39) strains of bacteria producing alkaline protease out of 57 strains were isolated from different soil samples. Based on clear zone obtained and production of protease under alkaline conditions, five strains were selected for further characterization. Strain MW05 and 08 showed maximum activity at pH 9.0 as compared to strains MW17, MW28 at pH 10 and strain MW09 at pH 8.0 (data not shown). These strains were grown in same medium as described in materials and methods, but at pH optimum for respective strains. Strain MW05 showed rapid growth in the initial phase with maximum cell mass (2.12 OD/ml) in 48 h (Figure 1). As compared to this, other strains are slow growing and less cell biomass were observed. Maximum production of 4675 U/ml was

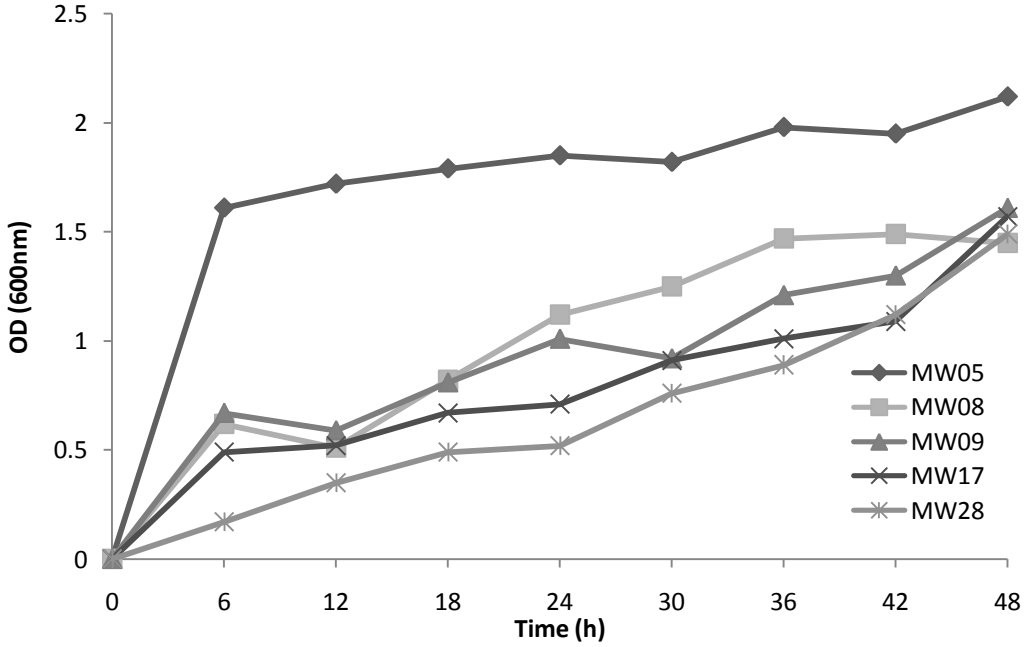

Figure 1. Growth profile of microorganisms producing alkaline proteases.

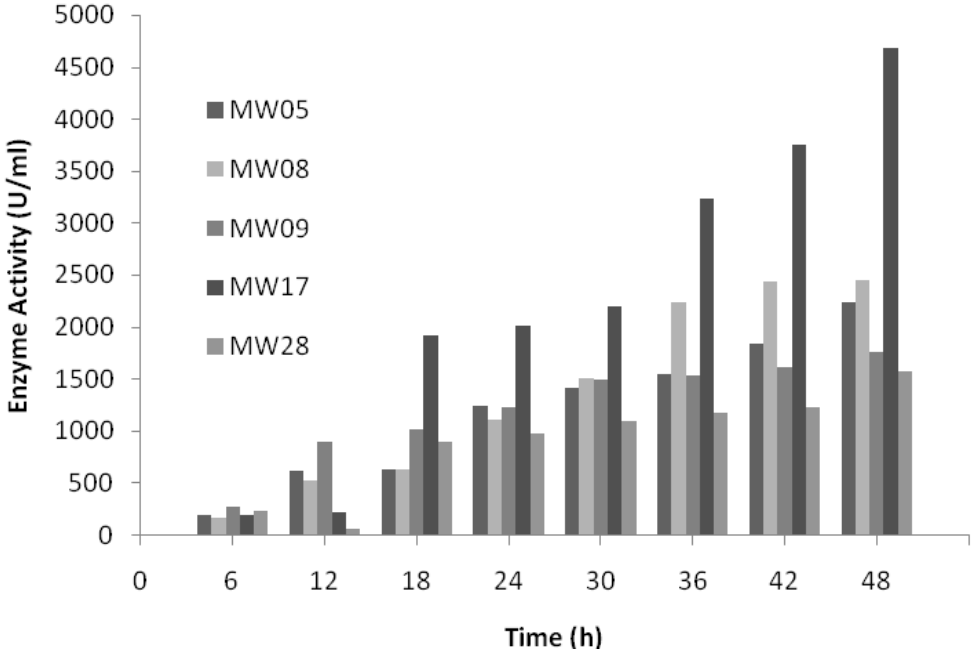

Figure 2. Enzyme activity of alkaline protease produced by different bacterial strains.

obtained with MW17 within 48 h, however, specific activity of 21515 U/mg protein was maximum with strain MW09. The production of protease was found to be growth associated in all the strains (Figures 1 and 2).

Characterization of enzyme

Partial purified enzymes from different strains were subjected to characterization studies. The result of pH

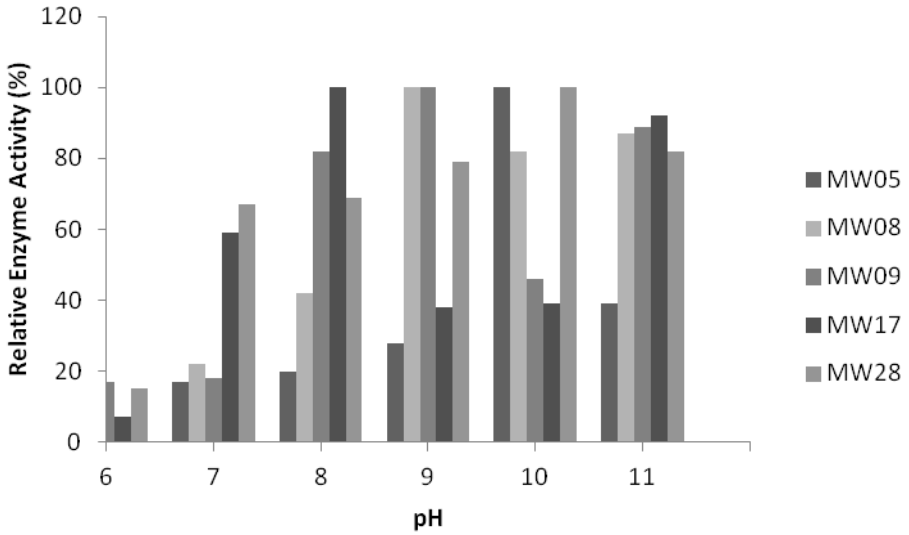

Figure 3. Effect of pH on the partially purified alkaline protease activity.

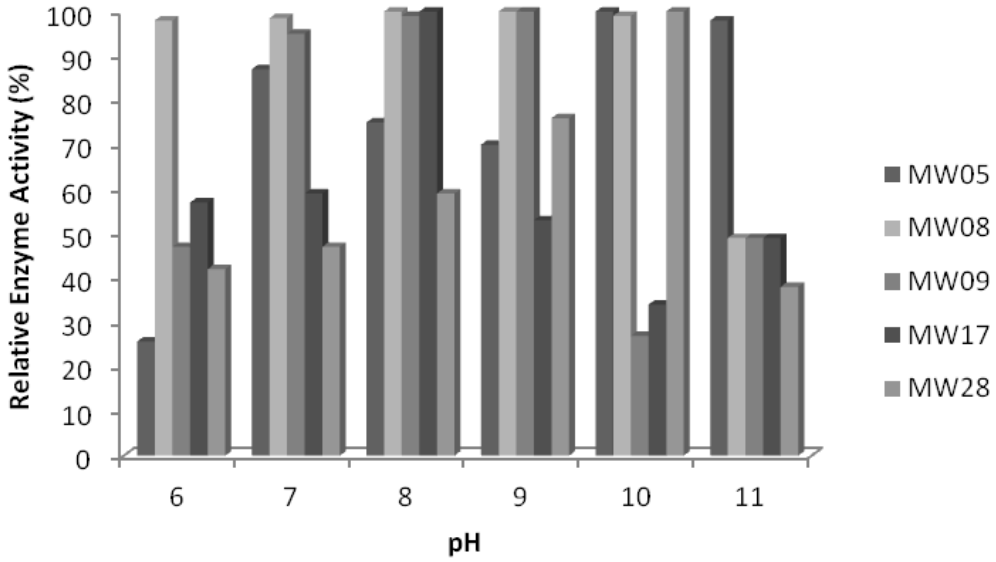

Figure 4. pH stability of alkaline protease produced by different bacterial strains.

studies indicated a broad pH activity range of 8.0-11.0 (Figure 3). Strain MW05 showed optimum pH of 10.0. This enzyme is sensitive to change in pH. As pH increased or decreased, the activity decreased drastically. However, in the case of MW28, smooth decrease was observed with change in pH. There was a special observation in MW09, there was two optimum at pH 9 and pH 11 with relative activity of 100 and 89%, respectively. At pH 8, about 82% of protease activity was observed as compared to the control. For MW17, pH optimum was at 8 and 11, thereby, indicating the possibility of isoenymes of protease in these organisms. Protease from MW08 showed optimum activity at pH 9. The optimum catalytic activity from different proteases reported is in the range of pH 7-11 (Cha et al., 2005; Kocabiyik and Ozdemir, 2006; Miyaji et al., 2006; Vidyasagar et al., 2006). The enzymes obtained from MW08 showed wide stability from pH 6-10 with more than 98% activity retained even after 24 h of incubation (Figure 4). About 87 and 98% protease activity was retained at pH 7 and 11 respectively, for MW05 protease. MW09 enzyme retained 95% of activity at pH 7-9 after 24 h

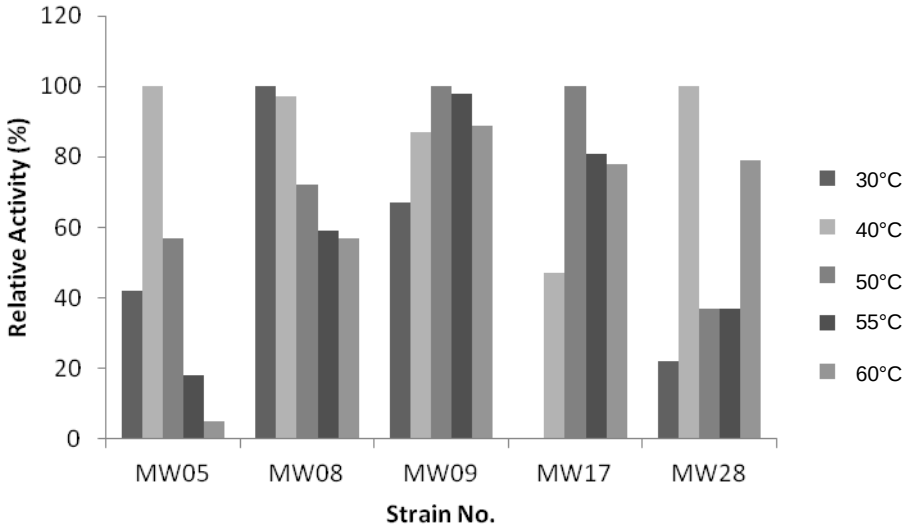

Figure 5. Effect of incubation temperature on alkaline protease produced by different bacterial strains.

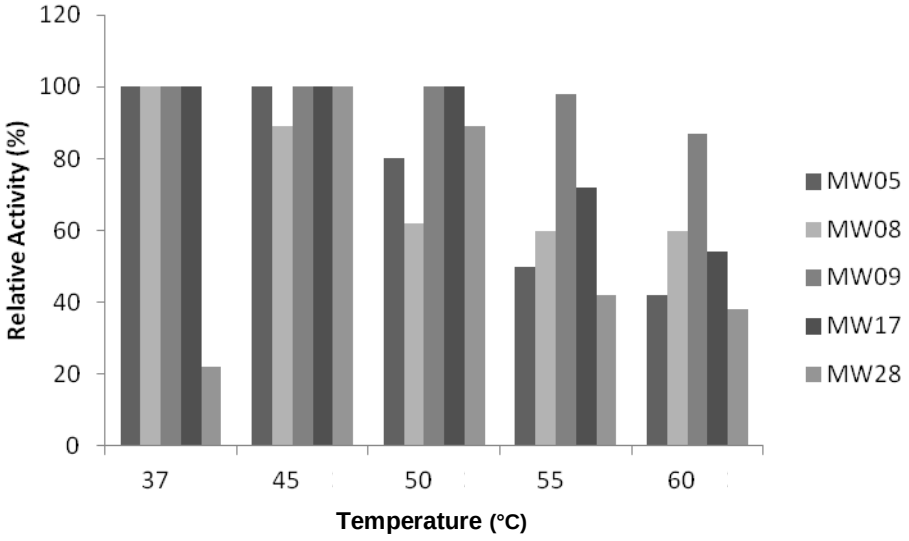

Figure 6. Stability of the protease enzyme from different strains.

of incubation, while 49% activity was retained at pH 11. MW28 protease had 100% retention in activity at its optimum pH of 10. In *Bacillus pumilus,* alkaline protease was stable over the pH 8-11 at 50°C (Miyaji et al., 2006).

The protease activity of the crude enzyme was measured at temperatures ranging from 30-60°C at different optimum pH: 8-10. MW05 and MW28 protease have 40°C to be the optimum temperature, while, MW08 strain had heat sensitive protease with optimum at 30°C. MW09 protease had optimum activity at 50°C, while 98 and 89% of relative activity was observed at 55 and 60°C, respectively. Partial purified MW17 protease activity decreased as temperature rose from 50 to 60°C (Figure 5). The optimum catalysis reported is in the range of 30 (Shikha et al., 2007), 37 (Patel et al., 2005) and 55°C (Huang et al., 2003). Our studies (Figure 6) indicated that strain MW09 protease was completely stable at temperature range of 37-50°C. Only 2% loss in activity was observed even after incubation at 55°C. This range of stability is quite broad as compared to some reports for alkaline proteases, where the stability is maximum at 50°C (Chu, 2007) and 55°C (Wang et al., 2006). Similarly, 100%

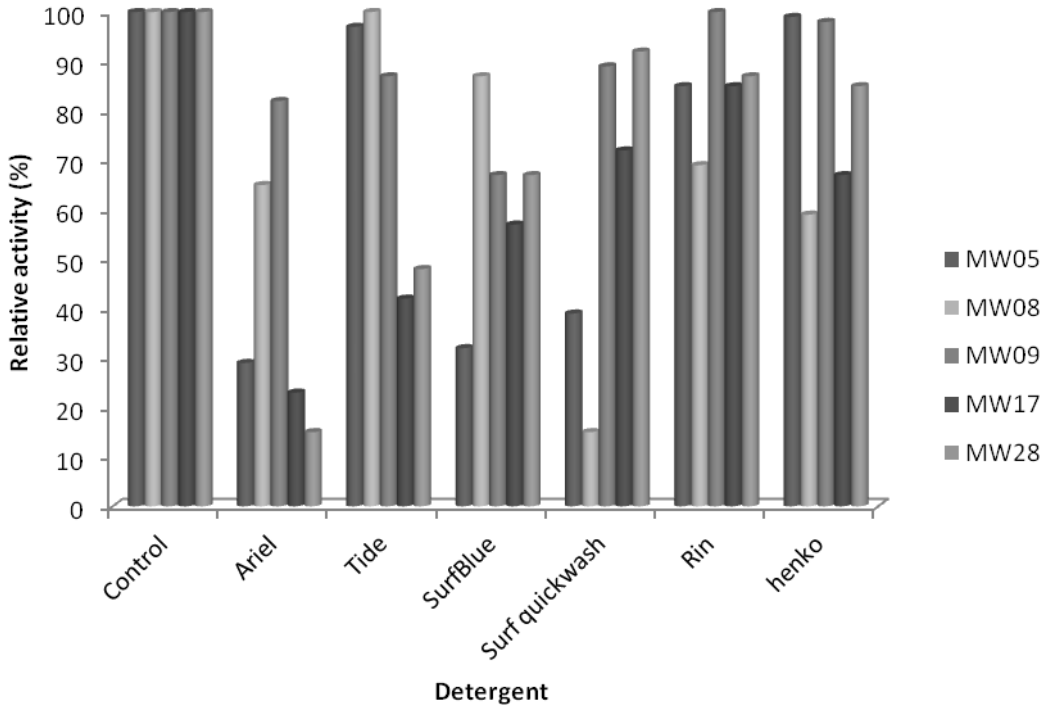

Figure 7. Stability of the protease enzyme in commercial detergents.

Table 1. Effect of various metal ions on protease activity.

Metal ion	Relative enzyme activity (%)				
	MW05	MW08	MW09	MW17	MW28
Ca^{2+} ($CaCl_2$)	149	15	110	57	5
Fe^{3+} ($FeCl_3$)	250	89	315	69	75
Cu^{2+} ($CuSO_4$)	157	97	105	100	100
Na^{+} (NaCl)	182	99	82	89	115
Mg^{2+} ($MgCl_2$)	197	99	110	215	87
Zn^{2+} ($ZnSO_4$)	167	97	69	217	120
Control	100	100	100	100	100

activity was retained even after incubation at 24 h upto 50°C in MW17 protease. Half life ($t_{1/2}$) of MW17 protease at 60°C was found to be 24 h.

A good detergent protease is expected to be stable in the presence of detergents. Wide variation in the stability of proteases isolated from different strains was observed (Figure 7). Strain MW08 showed complete stability in Tide. Protease MW05 was almost 98% stable in Tide and Henko detergent. MW09 protease had wide stability (67-98%) in varieties of detergents. Different proteases demonstrated variation in stability when applied in the presence of commercial detergents (Gupta et al., 2008; Vijayalakshmi et al., 2011). The effect of various metal ions on protease activity was tested in 50 mM glycine-NaOH buffer. Among the metal ions tested, Fe^{3+} acted as strong enhancer of enzyme with enhancement of activity upto 315% (Table 1). Zn^{2+}, Mg^{2+}, Na^{+} and Ca^{2+}enhanced the activity by 217 (MW17), 215 (MW17), 182 (MW05) and 149% (MW05) respectively. Ca^{2+} acted as strong inhibitor by decreasing activity to 5% of MW28 protease. As protease MW09 showed maximum desired properties (maximum production, optimum pH of 9 and 11; pH stability of 7-9; temperature optimum 55-60°C; temperature stability 37-55°C and stability in metal ions and detergents), it made it to be suitable for industrial applications. The strain MW09 was characterized with

Table 2. Layout and results in the GENIII Microplate™.

A1	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**	**A8**	**A9**	**A10**	**A11**	**A12**
Negative control	Dextrin	D-Maltose	D-Trehalose	D-Cellobiose	Gentiobiose	Sucrose	D-Turanose	Stachyose	Positive Control	pH 6	pH 5
-	+	+	+	-	-	±	±	-	+	±	±
B1	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**	**B8**	**B9**	**B10**	**B11**	**B12**
D-Raffinose	α-D-Lactose	D-Melibiose	β-methyl-D-Glucoside	D-Salicin	N-acetyl- β- D-Glucosamine	N-acetyl- β-D-Mannosamine	N-acetyl-D-Galactosamine	N-acetyl-Neuraminic acid	1% NaCl	4% NaCl	8% NaCl
-	-	-	+	+	±	-	-	-	+	+	+
C1	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C9**	**C10**	**C11**	**C12**
α-D-Glucose	D-mannose	D-Fructose	D-Galactose	3- methyl Galactose	D-Fucose	L-Fucose	L-Rhamnose	Inosine	1% Sodium lactate	Fusidic acid	D-Serine
+	+	+	±	-	-	-	±	-	+	-	-
D1	**D2**	**D3**	**D4**	**D5**	**D6**	**D7**	**D8**	**D9**	**D10**	**D11**	**D12**
D-Sorbitol	D-Mannitol	D-Arabitol	Myo-Inositol	Glycerol	D-Glucose-6-Phosphate	D-Fructose-6-Phosphate	D-Aspartic acid	D-Serine	Troleandomycin	Rifamycin SV	Minocycline
±	±	-	±	-	-	-	±	-	-	-	-
E1	**E2**	**E3**	**E4**	**E5**	**E6**	**E7**	**E8**	**E9**	**E10**	**E11**	**E12**
Gelatine	Glycine-L-Proline	L-Alanine	L-Arginine	L-Aspartic acid	L-Glutamic acid	L-Histidine	L-pyro Glutamic acid	L-Serine	Lincomycin	Guanidine HCl	Niaproof-4
-	-	±	±	±	±	-	-	±	-	±	-
F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**	**F10**	**F11**	**F12**
Pectin	D-Galacturonic acid	L-Galactonic acid lactone	D-Gluconic acid	D-Glucuronic acid	Glucuronamide	Mucic acid	Quinic acid	D-Saccharic acid	Vancomycin	Tetrazolium Violet	Tetrazolium Blue
-	±	-	+	±	-	-	-	-	-	-	-
G1	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**	**G8**	**G9**	**G10**	**G11**	**G12**
p-hydroxyl phenyl acetic acid	Methyl pyruvate	D-Lactic acid methyl ester	L-Lactic acid	Citric acid	α-keto-Glutaric acid	D-Mallic acid	L-Mallic acid	Bromo-succinic acid	Nalidixic acid	Lithium Chloride	Potassium Tellurite
-	-	-	+	±	-	-	+	±	-	±	+
H1	**H2**	**H3**	**H4**	**H5**	**H6**	**H7**	**H8**	**H9**	**H10**	**H11**	**H12**
Tween -40	γ–amino butyric acid	α-hydroxy-butyric acid	β-hydroxy-D,L-butyric acid	α-keto-butryric acid	Aceto-acetic acid	Propionic acid	Acetic acid	Formic acid	Aztreonam	Sodium butyrate	Sodium bromate
±	±	-	-	-	-	-	-	-	+	±	-

- No growth, + growth, ± border line.

respect to physiological and biochemical properties and identified using BioLog system. The BioLog GEN III Microplate (table 2) was used which provided 71 carbon utilization assays, 23 chemical sensitivity assays and two controls (positive and negative). The microplate

data was entered and analyzed with MicroLog software. MW09 strain was identified as Gram positive rod shaped *Bacillus licheniformis* MW09. Further purification and characterization of protease from this strain is being carried out.

REFERENCES

Binod P, Palkhiwala P, Gaikaiwari R, Nampoothiri KM, Duggal A, Dey K, Pandey A (2013). Industrial enzymes-present status and future perspectives for India. J. Sci. Ind. Res. 72(5):271-286.

Cha M, Park JR, Yoon KY (2005). Purification and characterization of an alkaline serine protease producing angiotensin I-converting enzyme inhibitory peptide from *Bacillus sp*. SS103. J. Med. Food 8(4):462-468.

Chi Z, Ma C, Wang P, Li HF (2007). Optimization of medium and cultivation conditions for alkaline protease production by the marine yeast *Aureobasidium pullulans*. Bioresour.Technol. 98:534-538.

Chu WH (2007). Optimization of extracellular alkaline protease production from species of *Bacillus*. J. Ind. Microbiol. Biotechnol. 34:241-245.

Gupta A, Joseph B, Mani A, Thomas G (2008). Biosynthesis and properties of an extracellular thermostable serine alkaline protease from *Virgibacillus pantothenticus*. World J. Microbiol. Biotechnol. 24(2):237-243.

Huang Q, Peng Y, Li X, Wang H, Zhang Y (2003). Purification and characterization of an extracellular alkaline serine protease with dehairing function from *Bacillus pumilus*. Curr. Microbiol. 46(3):169-173.

Khan F (2013). New microbial proteases in leather and detergent industries. Innov. Res. Chem. 1:1-6.

Kocabiyik S, Ozdemir I (2006). Purification and characterization of an intracellular chymotrypsin-like serine protease from *Thermoplasma volcanium*. Biosci. Biotechnol. Biochem. 70(1):126-134.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193(1):265-275.

Miyaji T, Otta Y, Nakagawa T, Watanabe T, Niimura Y, Tomizuka N (2006). Purification and molecular characterization of subtilisin-like alkaline protease BPP-A from *Bacillus pumilus* strain MS-1. Lett. Appl. Microbiol. 42(3):242-247.

Moreira KA, Albuquerque BF, Teixeira MFS, Porto ALF, Lima Filho JL (2002). Application of protease from *Nocardiopsis sp*. as a laundry detergent additive. World J. Microbiol. Biotechnol. 18(4):309-315.

Najafi MF, Deobagkar D, Deobagkar D (2005). Potential application of protease isolated from *Pseudomonas aeruginosa* PD100. Elect. J. Biotechnol. 2:8197-8203.

Nigam PS (2013). Microbial enzymes with special characteristics for biotechnological applications. Biomolecules 3(3):597-611.

Ogawa J, Shimizu S (1999). Microbial enzymes: new industrial applications from traditional screening methods. Trends Biotechnol. 17(1):13-21.

Patel R, Dodia M, Singh SP (2005). Extracellular alkaline protease from a newly isolated haloalkaliphilic *Bacillus sp.:* Production and optimization. Process Biochem. 40:3569-3575.

Ray A (2012). Protease enzyme-potential industrial scope. Int. J. Tech. 2:01-04.

Sathiya G (2013). Production of protease from *Bacillus subtilis* and its application in leather making process. Int. J. Res. Biotechnol. Biochem. 3(1):7-10.

Shaginian KA, Alekhina IA, Denisova NP (1990). Serine proteinase from the higher basidiomycetes of *Coprinus* genus. Biokhimia 55:1387-1395.

Shikha, Sharan A, Darmwal NS (2007). Improved production of alkaline protease from a mutant of alkalophilic *Bacillus pantotheneticus* using molasses as a substrate. Bioresour. Technol. 98:881-885.

Singh J, Batra N, Sobti RC (2001). Serine alkaline protease from a newly isolated *Bacillus sp.* SSR1. Process Biochem. 36:781-785.

Vidyasagar M, Prakash SB, Sreeramulu K (2006). Optimization of culture conditions for the production of haloalkaliphilic thermostable protease from an extremely halophilic archaeon *Halogeometricum sp.* TSS101. Lett. Appl. Microbiol. 43:385-391.

Vijayalakshmi S, Venkatkumar S, Thankamani V (2011). Screening of alkalophilic thermophilic protease isolated from *Bacillus* RV.B2.90 for Industrial applications. Res. Biotechnol. 2(3):32-41.

Wang M, Yang J, Zhang KQ (2006). Characterization of an extracellular protease and its cDNA from the nematode-trapping fungus *Monacrosporium microscaphoides*. Can. J. Microbiol. 52(2):130-139.

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Efficiency of new plant growth promoting rhizobacteria on corn diseases control

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The efficacy of two plant growth promoting rhizobacteria (PGPR) strains including *Bacillus subtilis* TU-Orga1 and *Pseudomonas fluorescens* TU-Orga2 obtained from rice rhizosphere against naturally occurring diseases as well as their capacity to improve crop yield of sweet corn cv. Insee2 was studied. TU-Orga1 was significantly greater in inhibition of *Acidovorax avenae* subsp. *avenae* (*Aaa*) and *Erwinia chrysanthemi* pv. *zeae* (*Ecz*), caused bacterial leaf streak and stalk rot of corn, respectively by antibiosis. Greenhouse experiments using TU-Orga1 and TU-Orga2 treatments increased highest salicylic acid accumulation in corn leaves with 7.85 and 6.98 μg g^{-1} fresh weight, respectively to protect *Ecz* infection. Each PGPR strain was single applied in the field through seed treatment and 3-foliar-spray-intervals at 14, 21, and 28 days after planting. Two PGPR treatments resulted in reduced severity of all diseases and increased yields when compared with the control treatment. There were differences among the treatments in that the highest level of disease suppression of bacterial stalk rot resulted with treatments TU-Orga1 (P = 0.05), whereas TU-Orga2 showed the highest level of disease suppression of bacterial leaf streak and sugarcane mosaic virus and provided significantly greater marketable yield increases than the other treatments. This illustrates the potential of these new biocontrol agents to suppress multiple diseases. They could become a component of an integrated program or an organic farming for corn disease management.

Key words: Plant growth promoting rhizobacteria (PGPR), *Acidovorax avenae* subsp. *avenae, Erwinia chrysanthemi* pv. *zeae*, SCMV, systemic acquired resistant.

INTRODUCTION

Corn is an economically important crop and many disease-causing organisms including several fungi, viruses, bacteria, and nematodes disseminated in the corn production system. Increased production of corn has led to emerging disease problems of various diseases including seed and seedling diseases, downy mildew, Northern corn leaf blight, Southern corn leaf blight, Stewartii's wilt, bacterial leaf streak, bacterial stalk rot, and sugarcane mosaic virus (SCMV). Seed and seedling diseases caused by *Pythium* sp., *Rhizoctonia* sp. and *Fusarium* sp., downy mildew caused by *Peronosclerospora sorghi*, Northern corn leaf blight caused by *Setosphaeria turcica*, Southern corn leaf blight caused by *Bipolaris maydis*, bacterial leaf streak caused by *Acidovorax avenae* subsp. *avenae* (*Aaa*), and bacterial stalk rot caused by *Erwinia chrysanthemi* pv. *zeae* (*Ecz*) have occurred in many corn growing areas in Thailand (Prathuangwong et al., 2004). Also, SCMV is one of the most important viruses

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infecting corn and the symptoms include necrosis and blight on leaf, stem, flower, and ear (Li et al., 2000). These diseases are causing severe economic loss of corn production in Thailand, so effective control measures are critical. Although chemicals are available for the management of corn diseases, inappropriate and non-discriminative use of chemicals is known to cause undesirable effects such as residual toxicity, development of resistance, environmental pollution, health hazards to humans and animals, increased expenditure for plant protection and are inefficient in controlling viral disease. Based on the fact that there are no direct control measures, management through induction of plants natural defense is important and sustainable. In recent years, plant defense induction have been extensively evaluated as a mean to defense themselves against pathogens based on the systemic acquired resistance (SAR) (Ryals et al., 1996; van Loon, 1997; Sticher et al., 1997; Vallad and Goodman, 2004). The inducers could be either synthetic compounds and biological agent provides an induced resistance to a broad range of pathogens. Plant defense induction by biological agent or plant growth promoting rhizobacteria (PGPR) has been established against Cucumber mosaic virus, Tobacco mosaic virus, and Tomato mottle virus (Maurhofer et al., 1998; Murphy et al., 2000; Raupach et al., 1996). Also, several PGPR strain including *Bacillus amyloliquefaciens* KPS46 and *Paenibacillus pabuli* SW01/4 have been reported as offering several functions including increased plant growth by indole-3-acetic acid induction and induced plant defense by SAR against multiple diseases (caused by fungi, bacteria, and viruses) in various crops such as soybean, rice, sesame, corn, sunflower, and vegetable (Prathuangwong and Kasem, 2004; Boonnadakul et al., 2012; Sathitthampana et al., 2012; Athinuwat, 2013). Existing control measures are inadequate for commercial production, while biological control using PGPR has become more important. The present study evaluated the efficacy of *Bacillus subtilis* TU-Orga1 and *P. fluorescens* TU-Orga2 isolated from rice rhizosphere to increase growth and vigor of the sweet corn plant and thereby control bacterial leaf streak, bacterial stalk rot, and other naturally disease infections via salicylic accumulation pathway. Biological control using PGPR in a system of integrated control measures may provide effective and sustainable management where chemical control is not available or practical.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Two new plant growth promoting rhizobacteria (PGPR) strains, *Bacillus subtilis* TU-Orga1 and *Pseudomonas fluorescens* TU-Orga2 isolated from rice rhizosphere and two commercial PGPR strains, *Bacillus amyloliquefaciens* KPS46 and *Paenibacillus pabuli* SW01/4 that have been isolated from soybean rhizosphere (Prathuangwong and Kasem, 2004) were evaluated. In addition, virulent strains of *Acidovorax avenae* subsp. *avenae* (*Aaa*), causal agent of bacterial leaf streak and *Erwinia chrysanthemi* pv. *zeae* (*Ecz*), causal agent of bacterial stalk rot were used in this study. Each strain stored in 50% glycerol at -80°C was reactivated by streaking onto nutrient agar (NA) plate (5 g of bacto-peptone, 3 g of beef extract, 15 g of agar, and 1 L of H_2O) and incubated at room temperature (28 ± 2°C) for 48 h. A loopful of each culture strain was transferred to nutrient broth (NB) containing 5 g of bacto-peptone, 3 g of beef extract, and 1 L of H_2O and incubated on a rotary shaker at 150 rpm and at room temperature (28±2°C) for 48 h. Cells were harvested by centrifugation for 20 min at 4,000 g washed by a second centrifugation in sterile water and finally resuspended in sterile water to a final concentration of 10^6 - 10^8 cfu/ml prior to application.

Efficacy of plant growth promoting rhizobacteria (PGPR) against bacterial leaf streak and bacterial stalk rot pathogens

Inhibition of *A. avenae* subsp. *avenae* and *E. chrysanthemi* pv. *zeae* caused bacterial leaf streak and bacterial stalk rot of corn, respectively by PGPR strains *B. subtilis* TU-Orga1, *P. fluorescens* TU-Orga2, *B. amyloliquefaciens* KPS46, and *P. pabuli* SW01/4, were performed by paper disc diffusion assay to verify whether 4 PGPR strains exhibited target disease suppression before further greenhouse and field studies. They were a total of 12 treatments arranged in completely randomized design (CRD) with 10 replications. The PGPR cell suspension was grown overnight in 50 ml NB at room temperature (28 ± 2°C) and adjusted in sterile distilled water to 10^8 cfu/ml. The 10 µl of each bacterial antagonist suspension was dropped onto 5 mm diameter of sterile filter paper disc and was plated on NA amended with 1 ml of each pathogen suspension (10^8 cfu/ml). The inhibition zone was determined after incubation for 48 h at room temperature (28 ± 2°C). The experiment was conducted three times. The data were subjected to analysis of variance with the general linear models procedure of SAS program. Treatment means were assessed using Duncan's Multiple Range Test (DMRT) and all tests of significance were conducted at P = 0.05.

Efficacy of plant growth promoting rhizobacteria (PGPR) against bacterial stalk rot under greenhouse conditions

This experiment was conducted to test the efficacy of PGPR strains for enhancement of corn seedling growth. Corn seeds cv. Insee2 were surface disinfected by treatment with 95% ethanol (v/v) for 2 min, and washed with sterile distilled water 5 times in order to remove the ethanol. Before planting, 20 g of corn seeds were mixed thoroughly with 1 ml of each PGPR suspension (10^6 cfu/ml). Seeds treated by distilled water or copper hydroxide served as the negative and positive controls, respectively. They were a total of 14 treatments arranged in randomized complete block design (RCBD) with five replications per treatment (Table 1). The experiment was conducted three times. Treated seeds were planted in 30 cm-diameter pots (two seeds per pot) containing steam-pasteurized potting media (silt clay loam soil and sand mixed in equal volumes). The pots were kept under greenhouse conditions and watered daily. At seven days after seedling emergence, seedlings were harvested for measurement of root and shoot lengths and seed germination. Fresh cultures of all 4 PGPR strains were used as the target microorganisms for controlling bacterial stalk rot. *E. chrysanthemi* pv. *zeae* challenged inoculation at seven days after planting by toothpick inoculation technique were used (Prathuangwong et al., 2004). The incidence and disease reduction of bacterial stalk rot infection was determined and recorded at 3 to seven days after inoculation (Prathuangwong et al., 2004). The data were subjected to analysis of variance with the general linear models procedure of SAS program. Treatment means were assessed using DMRT and all tests of significance were conducted

Table 1. Plant growth promoting rhizobacteria strains application to suppress naturally corn disease infections under field conditions.

Code	Treatment[1/]
T1	Seed treatment with *Bacillus amyloliquefaciens* KPS46 (A)
T2	Seed treatment with *Paenibacillus pabuli* SW01/4 (B)
T3	Seed treatment with *Pseudomonas fluorescens* TU-Orga2 (C)
T4	Seed treatment with *Bacillus subtilis* TU-Orga1 (D)
T5	3-foliar-spray-intervals with KPS46 at 14, 21, and 28 days after planting (E)
T6	3-foliar-spray-intervals with SW01/4 at 14, 21, and 28 days after planting (F)
T7	3-foliar-spray-intervals with TU-Orga2 at 14, 21, and 28 days after planting (G)
T8	3-foliar-spray-intervals with TU-Orga1 at 14, 21, and 28 days after planting (H)
T9	A + E
T10	B + F
T11	C + G
T12	D + H
T13	3-foliar-spray-intervals with copper hydroxide and insecticide at 14, 21, and 28 days after planting
T14	Nontreated

[1/] *Bacillus subtilis* TU-Orga1 and *Pseudomonas fluorescens* TU-Orga2 are new biological control agents isolated from rice rhizosphere. *Bacillus amyloliquefaciens* KPS46 and *Paenibacillus pabuli* SW01/4 are commercial strains isolated from soybean (Prathuangwong and Kasem, 2004).

at P = 0.05.

Plant biochemical response analysis

At 5, 6, 7, 8, 9, 10, 11, and 12 days after planting, the treated plant under greenhouse conditions, the lower and the upper leaves of sweet corn were separately collected from each treatment in each day as mention above for SAR relating biochemical analysis. After being detached, each leaf was cut into 2 halves and pooled as 1 sample. Subsequently, leaf tissues were analyzed for salicylic acid (SA) accumulation by the method as described by Raskin et al. (1989). Pooled leaf tissue (0.5 g) from each replication were randomly sampled, frozen with liquid nitrogen and macerated in a cold mortar with 1 ml of extraction solution (90:9:1 volume of absolute methanol, glacial acetic acid, and distillate water). The extract was subsequently centrifuged at 12,000 *g* and 4°C for 15 min. and the supernatant was collected for the analysis. To determine the SA content, 500 µl of the supernatant was mixed with an equal volume of 0.02 M ferric ammonium sulfate, incubated at 30°C for 5 min, and the absorbance at 530 nm was read by a spectrophotometer. The read absorbance was subsequently compared to those of the reference standard to obtain the actual amount of SA in the sample.

Efficacy of plant growth promoting rhizobacteria (PGPR) to suppression of naturally occurring diseases infection

Four PGPR strains, *B. subtilis* TU-Orga1, *P. fluorescens* TU-Orga2, *B. amyloliquefaciens* KPS46, and *P. pabuli* SW01/4, known to be effective were studied for their efficacy to suppress naturally occurring diseases infection. The investigation had been carried out in Pathumthani province with a history of bacterial stalk rot and bacterial leaf streak during July-October, 2012. There were a total of 14 treatments arranged in RCBD with three replicate plots (Table 1). Plots of 5 × 7 m^2 separated by 0.5 m paths were grown with sweet corn in 50 × 50 cm spacing. Corn seeds (cv. Insee2) were separately grown in seedling pots. They were irrigated by furrow system 1 time/week. Within the cropping season, the naturally infected diseases on tested plants were controlled by seed treatment alone with each PGPR strain, 3-foliar-spray-intervals alone with each PGPR strain at 14, 21, and 28 days after planting, and seed treatment plus 3-foliar-spray-intervals at 14, 21, and 28 days after planting with each PGPR strain as treatment described in Table 1. Seed treatment used PGPR suspension concentration at 10^6 cfu/ml and foliar spray used PGPR cell suspension concentration at 10^8 cfu/ml. The potential of disease control was compared with agrochemical treatment, copper hydroxide plus insecticide as positive control treatment and nontreated treatment.

Data collection and analysis

The improvement of yield in term of quantity and quality and all disease incidences were measured in tested plants. Seed germination was evaluated at 14 days after planting. Yield production was measured at 90 days after planting with total and marketable yields by random sampling with 30 plants/ treatment. The incidence severity and reduction of all disease infection was determined and recorded at 60 days after planting.

RESULTS

Efficacy of plant growth promoting rhizobacteria (PGPR) against bacterial leaf streak and bacterial stalk rot pathogens

Relationship between *A. avenae* subsp. *avenae* and *E. chrysanthemi* pv. *zeae* suppression and production of antimicrobial compounds by *B. subtilis* TU-Orga1, *P. fluorescens* TU-Orga2, and *B. amyloliquefaciens* KPS46 was analyzed using a paper disc diffusion method where *P. pabuli* SW01/4 showed competition mode inhibition of

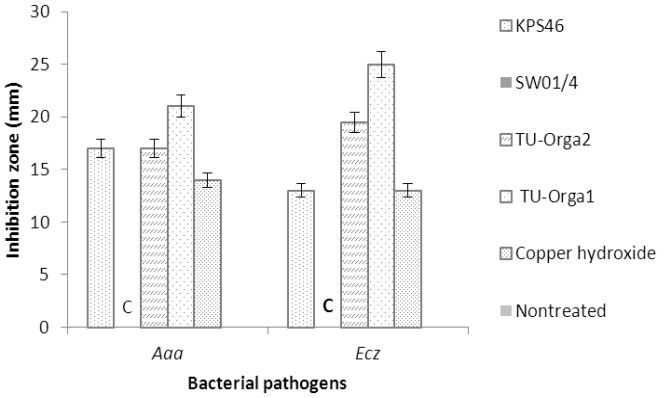

Figure 1. *Acidovorax avenae* subsp. *avenae* (*Aaa*) and *Erwinia chrysanthemi* pv. *zeae* (*Ecz*) growth inhibited by *Bacillus amyloliquefaciens* KPS46, *Paenibacillus pabuli* SW01/4, *Pseudomonas fluorescens* TU-Orga2, and *B. subtilis* TU-Orga1. C = competition, exhibited rapid growth, covered and inhibited the pathogen colonies.

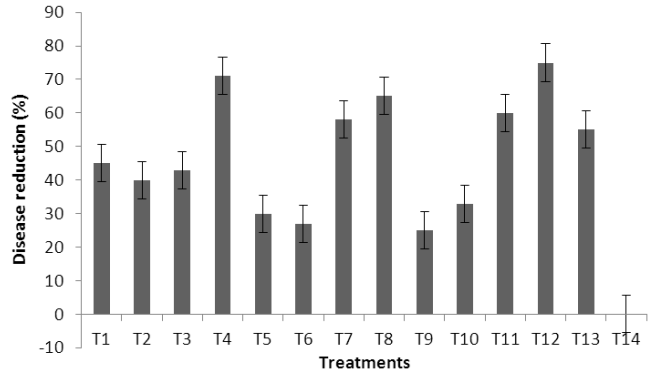

Figure 2. Efficacy of plant growth promoting rhizobacteria to control bacterial stalk rot of corn evaluated at 12 days after planting under greenhouse conditions. Codes T1 to T14 are shown in Table 1.

both pathogens by the same method (Figure 1). *B. subtilis* TU-Orga1 cells strongly suppressed growth of *A. avenae* subsp. *avenae* and *E. chrysanthemi* pv. *zeae* on NA plates, as revealed by a wide inhibition zone around the culture paper discs (Figure 1). For competition mode, *P. pabuli* SW01/4 exhibited rapid growth, covering and inhibiting the colonies of *A. avenae* subsp. *avenae* and *E. chrysanthemi* pv. *zeae.* These results demonstrate the different action modes and specificity modes of the 4 PGPR strains to bacterial leaf streak and bacterial stalk rot suppression.

Efficacy of plant growth promoting rhizobacteria (PGPR) against bacterial stalk rot under greenhouse conditions

We determine in this study, all of PGPR strains significantly increased seed germination, shoot, and root length better than the chemical treatment (copper hydroxide) and nontreated controls. Interestingly, seed treated with *B. subtilis* TU-Orga1 and *P. fluorescens* TU-Orga2 showed significantly highest increases in shoot and root length, and seed germination (Table 2).

Furthermore, seed treated with two these PGPR cells were significantly reduced better than seed treated with copper hydroxide (Funguran®) in bacterial stalk rot suppression (Figure 2). Interestingly, seed treatment and 3-foliar-spray-intervals at 14, 21, and 28 days after planting with strain *B. subtilis* TU-Orga1 (T12) was significantly reduced higher than the other treatments in bacterial stalk rot suppression (P = 0.05) (Figure 2). The results were correlated with paper disc diffusion method, *B. subtilis* TU-Orga1 produces secondary metabolites which may have lead enhanced plant growth and induced systemic resistance against pathogens. When applied as a seed treatment, the antagonistic bacteria can also promote growth and induce resistance against several diseases of corn where foliar spray may directly kill the pathogens by antibiosis mode and/or induce resistance (Buensanteai et al., 2008a).

Extrapolating from the accumulated literature, these PGPR may have numerous mechanisms including antagonism against pathogens, alteration of nutrient availability, and direct interactions with plants (Buensanteai et al., 2008b). Therefore, the application method was a significant factor in biological control.

Plant biochemical response analysis

In this study, the PGPR strains were evaluated for their ability to induce defense responses and related chemicals to protect corn from *E. chrysanthemi* pv. *zeae*, bacterial stalk rot pathogen infection. Corn treatment with *B. subtilis* TU-Orga1 and *P. fluorescens* TU-Orga2 triggered increased accumulation of SA biochemical markers associated with induced resistance mainly after *E. chrysanthemi* pv. *zeae* inoculation. Our results indicate that in corn plants of cultivar Insee2 treated with *B. subtilis* TU-Orga1 and *P. fluorescens* TU-Orga2, SA level increased significantly five days after treatment and much more three days after challenge inoculation with *E. chrysanthemi* pv. *zeae*, reaching the maximum concentration of 6.98, and 7.85 µg g^{-1} fresh weight (Figure 3). By contrast, SA accumulation in nontreated and chemical-treated (T13), but pathogen-inoculated corn, was considerably lower (1.69 and 2.17 µg g^{-1} fresh weight).

Efficacy of plant growth promoting rhizobacteria (PGPR) to suppression of naturally occurring diseases infection

All PGPR treatments showed significant efficacy for

Table 2. Seedling vigor of corn seeds cv. Insee2 treated with plant growth promoting rhizobacteria under greenhouse conditions[1].

Treatment[2]	Plant growth parameter		
	Seed germination (%)	Root length (cm)	Shoot length (cm)
KPS46	89[b]	8.0[b]	4.5[c]
SW01/4	86[bc]	7.5b[c]	5.9[b]
TU-Orga2	100[a]	9.0[a]	5.8[b]
TU-Orga1	100[a]	9.5[a]	6.5[a]
Copper hydroxide	80[c]	7.0[c]	4.4[c]
Nontreated	80[c]	7.0[c]	4.5[c]

[1]Means in a column followed by the same letter are significantly different according to DMRT. Data collected 7 days after planting. [2]KPS46 = *Bacillus amyloliquefaciens*, SW01/4 = *Paenibacillus pabuli*, TU-Orga1 = *B. subtilis*, and TU-Orga2 = *Pseudomonas fluorescens*.

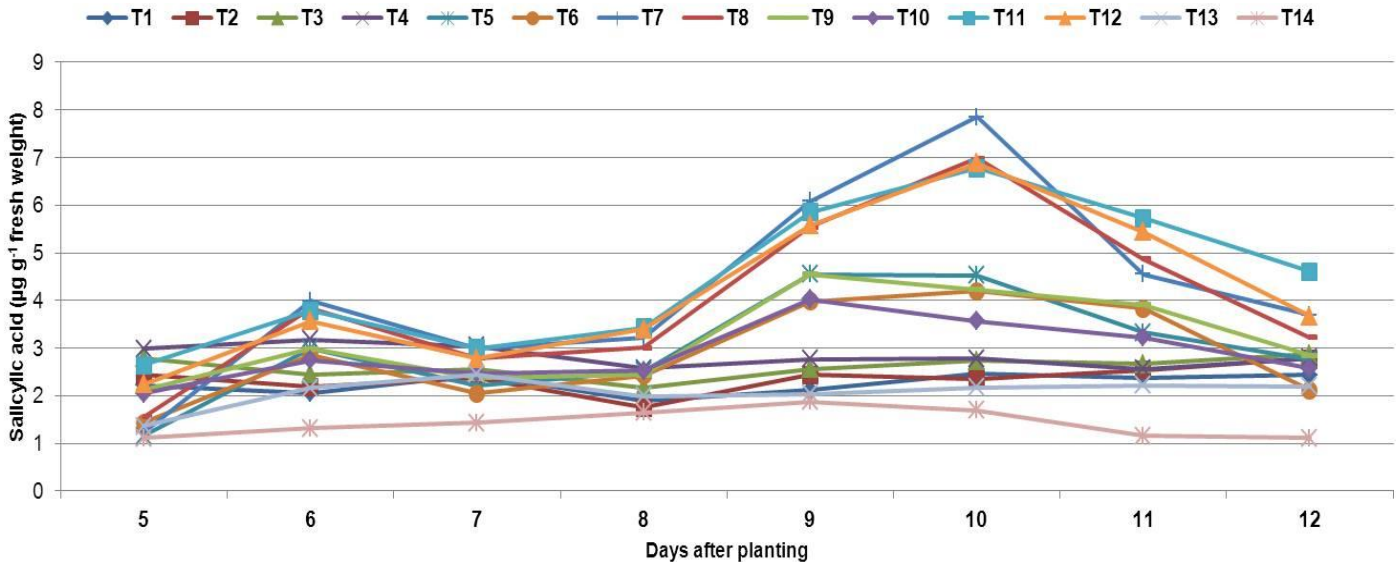

Figure 3. Accumulation of salicylic acid in leaves of sweet corn cultivars Insee2 pre- and post-challenge inoculation with *Erwinia chrysanthemi* pv. *zeae*. Codes T1 to T14 are shown in Table 1.

enhanced percentage of seed germination (Figure 4) and were significantly better than or equivalent to copper hydroxide (Funguran®) in reduced disease severity of bacterial leaf streak, bacterial stalk rot, and SCMV (Figure 5). There were differences among the treatments. The highest level of disease suppression of bacterial stalk rot resulted from treatments *B. subtilis* TU-Orga1 (T4, T8, T12) (P=0.05).

Interestingly, corn seeds treatments with *P. fluorescens* TU-Orga2 showed significantly highest increases in bacterial leaf streak and SCMV suppression (T3, T7, T11) (Figure 5). These PGPR strains appeared to have induced systemic resistance to various natural pathogens resulting in less infection compared with agrochemical, copper hydroxide (Funguran®) and nontreated control.

All treatments containing PGPR provided significant marketable yield increases (P=0.05) compared with that of chemical (Funguran®) and nontreated control (Figure 6). Interestingly, *P. fluorescens* TU-Orga2 provided greatest marketable yield increase and were significantly better in yield enhancement among the PGPR treatments (Figure 6). This indicates that PGPR enhanced plant growth, improved marketable yield, and induced resistance against bacterial stalk rot, bacterial leaf streak, and SCMV.

DISCUSSION

Many researchers have reported PGPR mediated plant protection against bacterial, fungal, and viral diseases of crop plants. In the present study, PGPR strains imparted beneficial properties in corn plants in terms of reduced disease severity and also resulted in significantly higher

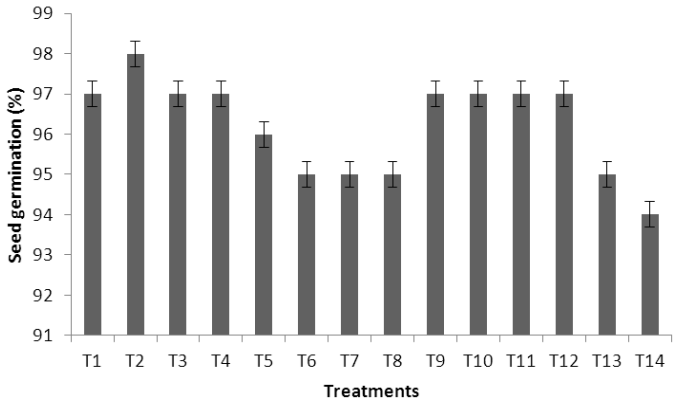

Figure 4. Efficacy of plant growth promoting rhizobacteria to enhances sweet corn cv. Insee2 seed germination at 14 days after planting under field conditions. Codes T1 to T14 are shown in Table 1.

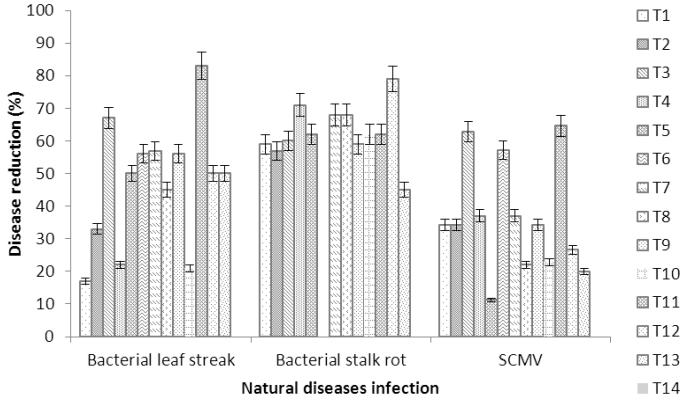

Figure 5. Efficacy of plant growth promoting rhizobacteria to reduction of bacterial leaf streak, stalk rot, and sugarcane mosaic virus diseases at 60 days after planting under field conditions. Codes T1 to T14 are shown in Table 1.

yield, when compared to the agrochemical (Funguran®) and nontreated treatment. Treatments with *B. subtilis* TU-Orga1, *P. fluorescens* TU-Orga2, *B. amyloliquefaciens* KPS46, and *P. pabuli* SW01/4 resulted in considerable disease suppression in corn plants.

The disease suppression observed in the PGPR treated plants is a product of antibiosis, enhanced plant growth, and induction of systemic resistance by the strains, as has been previously reported in many crops and also against many pathogen systems (Prathuangwong and Kasem, 2004; Sathitthampana et al., 2012). SAR by rhizobacteria has been proved against several bacterial, fungal, and viral plant-diseases (Alstrom, 1991; Leeman et al., 1995; Prathuangwong et al., 2005b; Sathitthampana et al., 2012).

B. amyloliquefaciens KPS46 and *P. pabuli* SW01/4 have been reported competitive with *Xanthomonas*

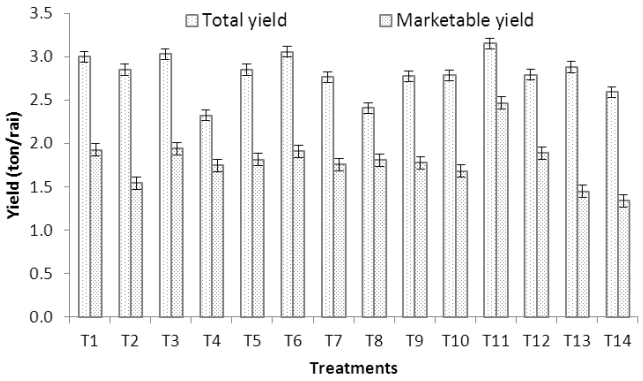

Figure 6. Efficacy of plant growth promoting rhizobacteria to enhances marketable yield of corn under field conditions. Codes T1 to T14 are shown in Table 1.

axonopodis pv. *glycines*, a soybean bacterial pustule pathogen. These two strains applied to seed treatment reduced various diseases of soybean in the field such as root rot (caused by *Fusarium* spp.), anthracnose (*Colletotrichum truncatum*), bacterial pustule (*X. axonopodis* pv. *glycines*), soybean crinkle leaf virus (SCLV), and soybean mosaic virus (SMV). They also increased yield quality and quantity through one or more mechanisms including competition (Prathuangwong and Kasem, 2004; Prathuangwong et al., 2005a; Buensanteai et al., 2009). Preecha et al. (2009) also reported that *B. amyloliquefaciens* KPS46 produced at least three effective antibiotics against *X. axonopodis* pv. *glycines*. Moreover, *B. amyloliquefaciens* KPS46 produced high levels of surfactin to inhibit *X. axonopodis* pv. *glycines*.

However, synthetic surfactants can stimulate plant growth by synergizing auxin action, activating certain plant enzyme systems, or affecting plant cell membrane permeability, thereby increasing water or nutrient uptake or excretion of plant factors such as riboflavin (Parr and Norman, 1965; Ernst et al., 1971).

B. subtilis TU-Orga1 has been reported competitive with *Xanthomonas oryzae* pv. *oryzae*, *Cercospora oryzae*, and *Bipolaris oryzae* the causal agent of bacterial blight, narrow brown spot, and brown spot diseases of rice, respectively (Boonnadakul et al., 2012; Sathitthampana et al., 2012). *B. subtilis* TU-Orga1 has been reported to secrete phytohormone like indole-3-acetic acid (IAA) for direct enhancement of rice growth (Sathitthampana et al., 2012). Therefore, secondary metabolites secreted by PGPR have a direct role in regulating plant growth, i.e., promote plant growth under optimal nutrient conditions and in the absence of plant deleterious organisms.

Bacteria and virus infection typically has a negative effect on photosynthesis and allocation of resources between organs, which leads to the characteristic chlorosis (Hull, 2002). A previously study has revealed the effect of the bacterial strain *B. amyloliquefaciens* KPS46 in bring-

ing out higher chlorophyll content in the leaves of the treated soybean plants (Buensanteai et al., 2009). This could be one of the reasons why there is a significantly higher yield in the *P. fluorescens* TU-Orga2 treated plants in the field. The present study shows 15 to 84% increases in the yield in the PGPR treated plants, when compared to the nontreated control.

The host resistance pathways involved in protection of crops from plant pathogen have been reported. Buensanteai et al. (2009) reported *B. amyloliquefaciens* KPS46 could express its function of salicylic (SA) and jasmonic acid (JA) signaling pathway to protect against *X. axonopodis* pv. *glycines*, soybean pathogen. This could be one of the reasons for *Bacillus* sp. significantly reduced *A. avenae* subsp. *avenae*, *E. chrysanthemi* pv. *zeae,* and SCMV infections. JA production by PGPR is responsible for the induction of systemic resistant in plants. SA is involved in systemic response related to defense processes, plays a key role in SAR response provoked by pathogen attack in many plant species, and treatment of SA will decrease the pathogen infection process (van Loon*,* 1997). Our study on the mechanism of biological control agents, two commercial strains *B. amyloliquefaciens* KPS46 and *P. pabuli* SW01/4 and new PGPR strains *B. subtilis* TU-Orga1 and *P. fluorescens* TU-Orga2 may be more than one mechanism to protect plant pathogen infection and enhance plant growth. With such information, we could potentially enhance the efficacy of biological control as a source of new bioproducts.

The host resistance pathways involved in protection of crops from viral diseases is unclear, even though there are various reports on systemic protection of plants from viral infection. Ahn et al. (2001) also suggest that there is simultaneous activation of both *PR-1a* and *PDF 1.2* genes in tobacco and *Arabidopsis* upon leaf-infiltration with EXTN-1. This indicated a SA and JA dependent pathway getting activated in crops with EXTN-1 as *PR-1a* gene is commonly used as an indicator of SA signaling and *PDF 1.2* of JA signaling (Reymond and Framer, 1998). Malamy et al. (1990) suggested SA is a likely endogenous signal in the resistance response of tobacco to viral infection.

Also, Ahn et al. (2001) demonstrated earlier the up regulation pathways of phenyl alanine ammonia lyase (PAL) and 3-hydroxy, 3-methylglutaryl CoA reductase (HMGR) in EXTN-1 treated tobacco plants upon challenge inoculation with Pepper mild mottle virus (PMMoV). Coordinated reduction of viral genome accumulation was clearly detected in the leaves of tobacco pretreated with EXTN-1. We have demonstrated earlier that *B. amyloliquefaciens* KPS46 has also induced production of SA, JA, PAL, peroxidase, and 1,3-β-glucanase indicating that these act as signals in the defense response pathway for the full expression of *PR-1a* and *PDF 1.2* genes and the other defense genes in *Arabidopsis* and soybean (Buensanteai et al., 2009). This could be one of the reasons why there is significantly lower SCMV infection in corn treated with PGPR strains. Furthermore, the mechanism of new PGPR strains *B. subtilis* TU-Orga1 and *P. fluorescens* TU-Orga2 need to be investigated.

This study reports on the use of PGPR with seed treatment and foliar spray for control of root and foliar diseases of corn. Crop management with an application of PGPR may provide longer term protection of plant development than that afforded by chemical (Funguran®) treatment alone. The data indicates that these PGPR strains have the potential for disease control and increasing plant growth and yield comparable to or superior to the pesticides and fertilizers for corn. All treatments using PGPR protected the plants satisfactorily against all natural disease infection, confirming the benefits of integrating control techniques. A biological control agent that can function in combination with other management tools to suppress diseases in the field might have value as a commercial product for disease control. Futuremore, for the Future perspective, the effect of environment, formulation and package on survival, stability and plant growth promoting ability of two new biological control agents will be studied.

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REFERENCES

Ahn IP, Park KS, Kim CH (2001). Rhizobacteria-induced resistance perturbs viral disease progress and triggers defense related gene expression. Mol. Cells 13:302-308.

Alstrom S (1991). Induction of disease resistance in common bean susceptible to halo blight bacterial pathogen after seed bacterization with rhizoshpere pseudomonads. J. Gen. Appl. Microbiol. 37:495-501.

Athinuwat D (2013). Beneficial microbes in agriculture. Thai J. Sci. Technol. 2(1):18-35.

Boonnadakul C, Chuaboon W, Athinuwat D (2012). Efficiency of mixed antagonistic strains to enhance organic rice growth and diseases control. Thai J. Sci. Technol. 1(3):189-196.

Buensanteai N, Athinuwat D, Chatnaparat T, Yuen GY, Prathuangwong S (2008a). Extracellular proteome of plant growth promoting-bacteria, *Bacillus amyloliquefaciens* KPS46 and its effect on enhanced growth promotion and induced systemic resistance on soybean. Kasetsart J. (Nat. Sci.). 42:13-26.

Buensanteai N, Yuen GY, Prathuangwong S (2008b). The biocontrol bacterium *Bacillus amyloliquefaciens* KPS46 produces auxin, surfactin and extracellular proteins for enhanced growth of soybean plant. Thai J. Agri. Sci. 41(3):101-116.

Buensanteai N, Yuen GY, Prathuangwong S (2009). Priming, signaling, and protein productivity associated with induced resistance by *Bacillus amyloliquefaciens* KPS46. World J. Biotechnol. 25:1275-1286.

Ernst RJ, Arditti, Healey PL (1971). Carbohydrate physiology of orchid

seedling. II. Hydrolysis and effects of oligosaccharides. Am. J. Bot. 58:827-835.
Hull R (2002). Matthews' Plant Virology. 4th ed. Academic Press, San Diego.
Leeman M, Van PJA, Den OFM, Heinsbroek M, Bakker PAHM, Schippers B (1995). Induction of systemic resistance by *Pseudomonas fluorescens* in radish cultivars differing in susceptibility to fusarium wilt, using a novel bioassay. Eur. J. Plant Pathol. 101:655-664.
Li XH, Han XQ, Zhang SH (2000). Research progress on maize dwarf mosaic virus. J. Maize 8:67-72.
Malamy J, Carr JP, Klessig DF, Raskin I (1990). Salicylic acid a likely endogenous signal in the resistance response of tobacco to viral infection. Science 250:1002-1004.
Maurhofer M, Reimmann C, Schmidli-Sacherer P, Heeb S, Haas D, Defago G (1998). Salicylic acid biosynthetic genes expressed in *Pseudomonas fluorescens* strain P3 improve the induction of systemic resistance in tobacco against tobacco necrosis virus. Phytopathology 88:678-684.
Murphy JF, Zehnder GW, Schuster DJ, Sikora EJ, Polston JE, Kloepper JW (2000). Plant growth-promoting rhizobacterial mediated protection in tomato against *Tomato mottle virus*. Plant Dis. 84:779-784.
Parr JF, Norman AG (1965). Considerations in the use of surfactants in plant systems: A review. Botanical Gazette 126:86-96.
Prathuangwong S, Juthawanthana P, Ketsuwan K, Kasem S (2004). An outbreak of new bacterial diseases of corn in Thailand. Proc. of the 1st Kasetsart University Corn and Sorghum Research Program Workshop, May 19 - 21, 2004. Ayutthaya, Thailand.
Prathuangwong S, Kasem S (2004). Screening and evaluation of thermotolerant epiphytic bacteria from soybean leaves for controlling bacterial pustule disease. Thai J. Agric. Sci. 37: 1-8.
Preecha C, Sadowsky MJ, Prathuangwong S (2009). Lipopeptide surfactin produced by *Bacillus amyloliquefaciens* KPS46 is required for biocontrol efficacy against *Xanthomonas axonopodis* pv. *glycines*. Kasetsart J. (Nat. Sci.). 44(1):84-99.
Raskin I, Turner I, Melander WR (1989). Regulation of heat production in the inflorescences of an Arum lily by endogenous salicylic acid. PNAS. 86:2214-2218.
Raupach GS, Liu L, Murphy JF, Tuzun S, Kloepper JW (1996). Induced systemic resistance in cucumber and tomato against cucumber mosaic cucumovirus using plant growth-promoting rhizobacteria (PGPR). Plant Dis. 80:91-894.
Reymond P, Framer EE (1998). Jasmonate and salycilate as global signals for defence gene expression. Curr. Opin. Plant Biol. 1:404-411.
Ryals JA, Neuenschwander UH, Willits MG, Molina A, Steiner HY, Hunt MD (1996). Systemic Acquired Resistance. Plant Cell 8:1809-1819.
Sathitthampana P, Chuaboon W, Athinuwat D (2012). Efficiency of new antagonistic bacterium TU-Orga1 enhanced growth and controlled bacterial leaf blight in rice plants. Thai J. Sci. Technol. 1(3):180-188.
Sticher L, Mauch-Mani B, Metraux JP (1997). Systemic acquired resistance. Annu. Rev. Phytopathol. 35:235-270.
Vallad GE, Goodman RM (2004). Systemic acquired resistance and induced systemic resistance in conventional agriculture. Crop Sci. 44:1920-1934.
Van LLC (1997). Induced resistance and the role of pathogenesis-related proteins. Eur. J. Plant Pathol. 103:753-765.

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African Journal of Microbiology Research

Short Communication

Isolation of antimicrobial producing lactobacilli from *akamu* (a Nigerian fermented cereal gruel)

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Accepted 30 January, 2014

Four samples of *akamu* were evaluated for the presence of antimicrobial producing lactobacilli. Thirty (30) lactobacilli were isolated from these samples. They inhibited the growth of *Staphylococcus aureus* and *Escherichia coli*, four of these isolated lactobacilli were chosen and when evaluated by agar well diffusion test, they inhibited the growth of the selected indicator organisms. The spectra of activity were evaluated against five different organisms. The highest activity was against *Proteus mirabilis* and the least against *Salmonella* sp.

Keywords: Bacteriocin, Lactobacilli, *akamu*, inhibition.

INTRODUCTION

In fermented foods, lactic acid bacteria have a long history of application because of their beneficial effects on nutritional, shelf-life and organoleptic characteristics of food. They cause acidification of the food through the production of organic acids, mainly lactic acid (Blandino et al., 2003; Omenu, 2011). The organic acids, bacteriocins, aroma compounds and several enzymes production are highly important (DeVuyst and Frederic, 2007; Afolabi et al., 2008). A food fermentation process with lactic acid bacteria is traditionally based on spontaneous fermentation. Lactobacilli exert strong antagonistic effects against many microorganisms, including food spoilage organisms and pathogens (Hartnett et al., 2002). They contribute to the preservation of foods by producing antimicrobial agents like bacteriocins which are considered as natural food preservatives (De Vuyst and Frederic, 2007; Cigden and Abamuslum, 2009; Asmahan and Muna, 2009). In *akamu*, lactic acid bacteria occur in large number and they confer qualities like extended shelf life, aroma and make the product safe for consumption.

Akamu is a lactic acid fermented gruel or porridge traditionally made from maize, millet and sorghum. It contributes substantially to the daily diet of both rural and urban communities especially as a weaning food for children (Adams, 1998) and food for invalids because it is light and digests easily (Afolayan et al., 2010). It is locally prepared on small scale in homes or for commercial purposes, and its quality may depend on the skills of the producers as inherited over the years (Chelule et al., 2010). The fermentation of *akamu* is spontaneous and mostly uncontrolled leading to products of variable quality. *Akamu* could be fortified with sugar, milk or chocolate to improve taste or sooth the sour taste. This study focused on the isolation of antimicrobial producing lactobacilli from *akamu*

MATERIALS AND METHODS

Isolation of lactic acid bacteria (LAB)

Fresh ready to sell *akamu* was bought from a local market in Nsukka. 10 ml of the sample was added to 90 ml of normal saline to make the initial dilution. This suspension was homogenized by

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Table 1. The lactobacilli isolated from *akamu*

A1	*L.acidophilus*
A2	*L.delbrueckii*
A3	*L.lactis*
A4	*L.sp*
A5	*L.casei*
A6	*L.acidophilus*
A7	*L.sp*
A8	*L.fermentum*
A9	*L.fermentum*
A10	*L.lactis*
A11	*L.delbrueckii*
A12	*L.casei*
A13	*L.lactis*
A14	*L.bulgaricus*
A15	*L.casei*
A16	*L.fermentum*
A17	*L.plantarum*
A18	*L.bulgaricus*
A19	*L.plantarum*
A20	*L.plantarum*
A21	*L.sp*
A22	*L.bulgaricus*
A23	*L.lactis*
A24	*L.bulgaricus*
A25	*L.acidophilus*
A26	*L.acidophilus*
A27	*L.sp*
A28	*L.delbrueckii*
A29	*L.lactis*
A30	*L.casei*

gentle manual agitation and serially diluted from 10^{-1} to 10^{-10}. Isolation of lactobacilli was done using pour plate method on de Mann Rogosa Sharpe (MRS). The plates were incubated in a micro aerophilic condition at 30°C. The incorporation of 0.02% sodium azide into the MRS agar made it selective for LAB.

Screening and selection of lactobacilli with antimicrobial activity

Initial screening and selection of lactobacilli colonies with antimicrobial activity was done using *Staphylococcus aureus, Escherichia coli* as target organisms. A total of thirty LAB organisms were isolated. The detection was done using 100 µl of overnight culture of the organisms inoculated in tryptic soy broth/yeast extract (with 0.7% agar), overlaid them on MRS plates on which the isolated lactobacilli colonies are grown. The inoculated plates were incubated at 37°C for 24 h. The lactobacillus organisms showing clear zones against the test organisms were considered as antimicrobial producers. The pure cultures of the isolates were stored in MRS broth and MRS agar slants and kept in the refrigerator. After incubation, based on the zones of inhibitions produced against the target organisms, four with greater inhibition zones were selected for further studies.

Bacteriocin production

The isolated lactobacilli were propagated in 1000 ml MRS broth at 30°C for 72 h under microaerophilic condition. For bacteriocin extraction, a cell-free solution was obtained by centrifuging the culture (10,000 rpm for 20 min), the culture was adjusted to pH 7 to remove the effect of organic acid and the effect of hydrogenperoxide was excluded by addition of catalase (C-100 bovine liver, sigma, United Kingdom) at a final concentration of 100 unit. The supernatant was then filtered through a 0.22 µm pore size membrane filter.

Antimicrobial activity of the bacteriocin

The antimicrobial activity of the isolates was done using the agar well diffusion method of Benkerroum et al. (1993). Sterile MRS agar plates were overlaid with 7 ml of MRS agar (0.7% agar) inoculated with 100 µl overnight broth cultures of indicator organisms, *E. coli, Proteus* sp*. S. aureus, Bacillus subtilis* and *Salmonella* sp. After solidifying, holes were bored with a cork borer on each plate; the holes were filled with 100 µl of the crude bacteriocins, allowed to diffuse and incubated, the antimicrobial activities were recorded as inhibition zone diameters.

RESULTS AND DISCUSSION

Isolation of antimicrobial producing lactobacilli from *akamu*

The lactic acid bacteria occurred in high frequency in the sample about 100 to 200 colonies per plate.

Identification of organisms

The isolated organisms were identified as *Lactobacillus acidophilus, Lactobacillus delbrueckii, Lactobacillus lactis* and *Lactobacillus* sp. using conventional microbiological methods according to Hammes and Hertel (2006) and Bergy's manual of systematic bacteriology (Table 1).

Agar well diffusion test

The selected *Lactobacillus* strains produced bacteriocin which showed inhibitory activity against the selected organisms. The bacteriocin had different profile of inhibition on the organisms. The largest spectrum of inhibition was shown by bacteriocin produced by *Lactobacillus lactis* and the least spectrum of inhibition was shown by that of *L. acidophilus*. The highest activity was shown against *Proteus* and the least against *Salmonella typhi* (Figures 1, 2, 3 and 4).

Conclusion

This research showed that antimicrobial producing

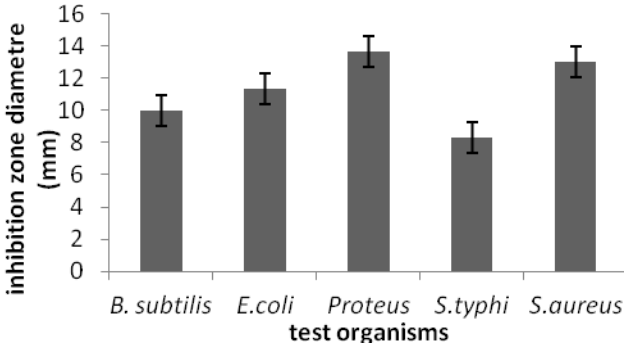

Figure 1. Agar well diffusion test for bacteriocin produced by *Lactobacillus acidophilus*.

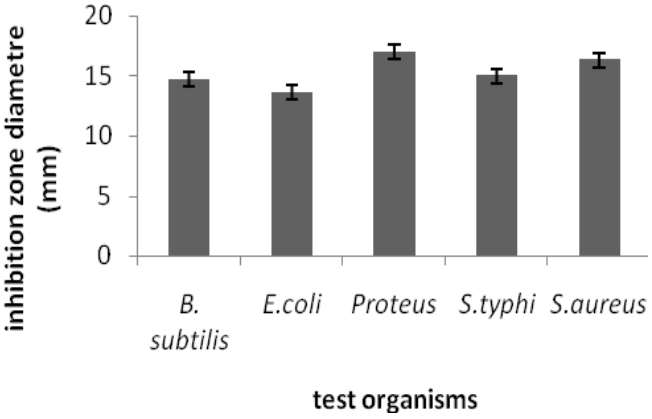

Figure 2. Agar well diffusion test for bacteriocin produced by *Lactobacillus delbrueckii*.

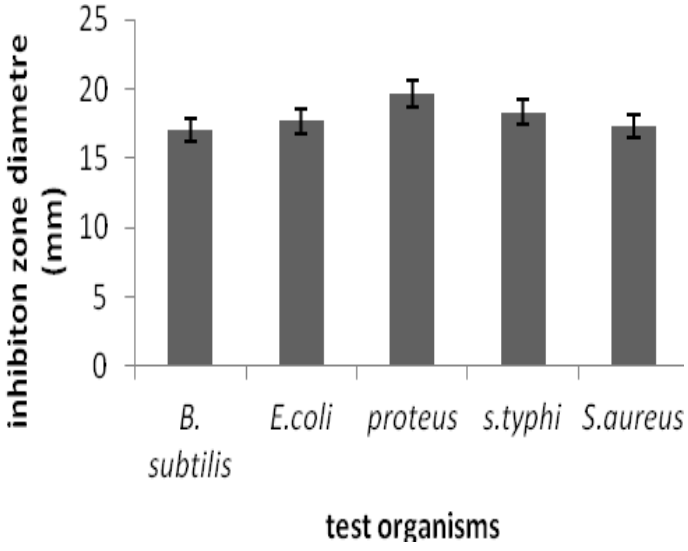

Figure 3. Agar well diffusion test for bacteriocin produced by *Lactobacillus lactis*.

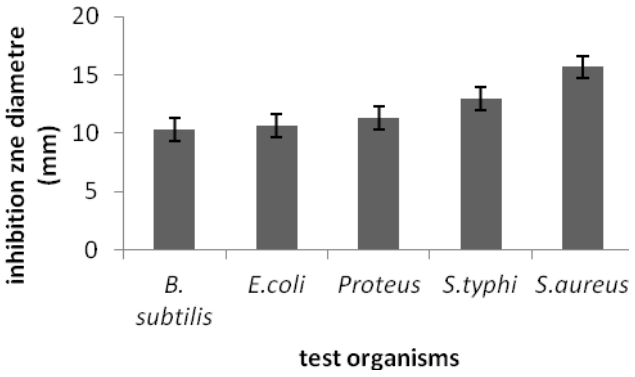

Figure 4. Agar well diffusion test for bacteriocin produced by *Lactobacillus* sp.

lactobacilli can be isolated from *akamu*. The agar well diffusion test further confirmed the inhibitory and antagonistic effects of these lactobacilli on pathogens and food spoilage organisms. The antagonistic effect is shown through the production of antimicrobial substances, e.g bacteriocin.

REFERENCES

Adams MR (1998). Fermented weaning foods, in J.B wood (Ed), Microbiology of fermented foods, pp. 790-811. London: BlackieAcademic .

Afolabi OR, Bankole OM, Olaitan OJ (2008). Production andcharacterization of antimicrobial agents by lactic acidbacteria isolated from fermented foods. Internet J. Microbiol. 4:2-6.

Afolayan MO, Afolayan M, Abuah JN (2010).An investigation into sorghum based ogi (ogi-baba) storage characteristics. Advanced J. Food Sci. Technol. 2:72-28.

Asmahan AA,Muna MM (2009). Isolation and identification of lacticacid bacteria from fermented sorghum dough used in Sudanesekisra preparation. Pakistan J. Nutr. 8: 1814-1818.

Benkerroum N, Ghouati Y, Sandine WE, Tantaoui-Elaraki A (1993). Methods to demonstrate the bactericidal activity of bateriocin. Lett. Appl. Microbiol. 17:80-81

Blandino A, Al-Aseeri ME, Pandiella SS, Cantero D,Webb C (2003).Cereal-based fermented foods and beverages. Food Res. Int. 36:527-543.

Chelule PK, Mbongwa HP, Carries S, Gqaleni N (2010b). Lactic acid fermentation improves the quality of amahewu, a traditional South African maize-based porridge, Food Chem. 122:656-661.

Cigden S, Abamuslum G (2009). Investigation of bacteriocinproduction capability of LAB isolated from food. Kafkas University Vet FakDerg15: 45-50.

De Vuyst L, Frederic L (2007). Bacteriocin from LAB: production, purification and food application. J. Mol. Microbiol. Biotechnol.13:194-199.

Hammes W, Hertel C (2006). The genera Lactobacilli and carnobacterium. Prokarotes 4: 320-403.

Hartnett DJ, Vaugan A, Van Sinderen D (2002). Antimicrobial producing Lactic acid bacteria isolated from raw barley and sorghum. Institute Guild Brewing108: 169-175

Omenu AM (2011). Fermentation dynamics during production of ogi, a Nigerian fermented porridge. Reports and Opinions 3: 8-17.

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Short Communication

HIV screening among patients followed for cryptococcal meningitis in developing countries: Data from Bukavu in the Democratic Republic of Congo

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We conducted a retrospective cohort study in the Departments of Internal medicine of 2 main Bukavu hospitals, in the South Kivu, DRC. The medical records of 7542 patients were reviewed. We observed 56 cases (11%) of cryptococcocal meningitis (CM) among 514 human immunodeficiency virus (HIV)-infected subjects. No case of CM was seen in HIV negative and 70 % of them had a concurrent diagnosis of tuberculosis as other active AIDS related diseases. Despite antifungal therapy, 90% of CM cases died.

Key words: Cryptococcal meningitis, HIV screening.

INTRODUCTION

In the low-income countries we can make the diagnosis of cryptococcocal meningitis (CM) on cerebro-spinal fluid (CSF) sample stained with India ink and examinated on light microscopy. Once this diagnosis is done, the main fear is that cryptococcocal meningitis remains one of major reason for HIV testing in some world's regions like in central Africa (Mbuagbaw et al., 2006).

MATERIALS AND METHODS

This retrospective cohort study focused on patients diagnosed with cryptococcocal meningitis (CM) in the two main hospitals of Bukavu, located in South Kivu province of the Democratic republic of Congo (DRC). This province is in the eastern DRC bordering Rwanda and Burundi. We reviewed inpatient hospital admissions at the Panzi Hospital (HGRP) and the Provincial Hospital of Bukavu (HPGRB). Panzi Hospital is a 350-bed general hospital for the local population, serving a significant population of sexual violence survivors. The population of Bukavu is approximatily 800,000 and the provincial hospital is the referral hospital for approximately 4.6 millions in the South Kivu province. We retrospectively reviewed 7542 medical records of hospitalized patients in the Departments of internal medicine over a 5 year period from May 1, 2007 to April 30, 2011. During this period, routine screening of HIV-infection occurred for all consenting patients admitted to hospital. The primary objective was to determine the rate of HIV positive tests among cryptococcocal meningitis (CM) cases. Secondary objectives were to determine the treatment received and the clinical outcomes. Analysis is primarily descriptive.

RESULTS

Among 7542 hospital admissions over a 5 year period, only 56 patients (0.7%) were hospitalized for cryptococcocal meningitis (CM) as diagnosed by India ink in light microscopy. Their mean age was 31.6 ± 2.9 years, and the

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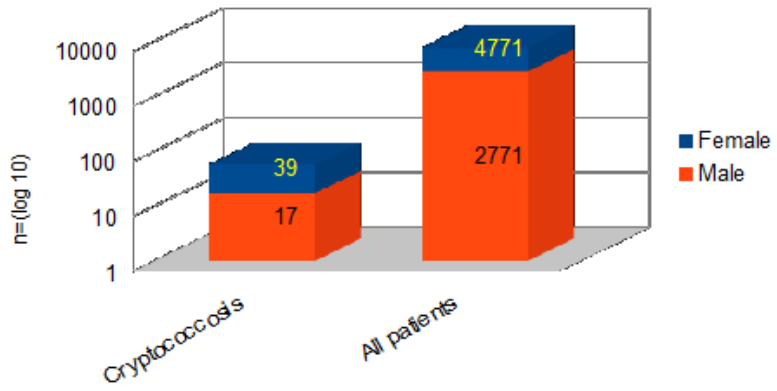


Figure 1. Histogram of the gender distribution.

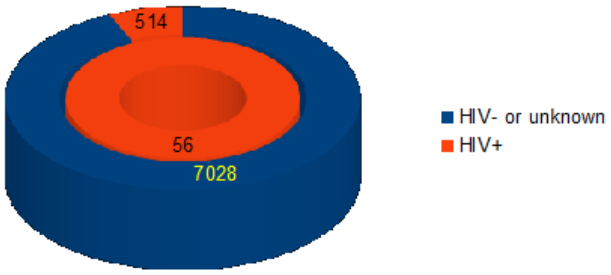


Figure 2. Histogram of the HIV screening.

Table 1. Symptoms associated with the meningeal cryptococcosis.

Symptom	At HGRP		At HPGRB	
	n	%	n	%
Headache	12	100	44	100
Weight loss	12	100	30	68
Confusion	4	33	38	86
Vomiting	5	42	25	57
Meningismus	12	100	44	100
Skin hyperesthesia	6	50	27	61
Prolonged fever	8	67	35	80

the *sex ratio* of women to men was 2:1 (Figure 1). All 56 patients with CM were screened HIV positive. But, among all hospital admissions, 6.4 % (n=514) were HIV-infected (Figure 2). Thus, cryptococcocal meningitis accounted for 11% of all hospital admissions for HIV-infected persons (95% CI: 8.2 to 13.6%). The most common symptoms present in persons with CM were weight loss (100%) and meningeal syndrome (100%) (Table 1). Concurrent *Mycobacterium tuberculosis* infection (TB) was diagnosed in 70% of those with CM cases (Table 2). The main antifungal therapy received was fluconazole alone in 82% (46/56) or others drugs that each patient received (Table 3). Overall, the in-hospital mortality rate in the CM group was 90% (50/56) (Figure 3).

DISCUSSION

In a five year period from 2007 to 2011, 56 hospitalized patients were diagnosed with cryptococcocal meningitis (CM) in the two main hospitals of Bukavu in the eastern DRC, accounting for 11 % of hospital admissions of HIV-

Table 2. Other AIDS related diseases associated with the meningeal cryptococcosis.

Aids related diseases	At HGRP		At HPGRB		All patients	
	n	%	n	%	n	%
Oral candidiasis	2	17	10	23	12	21
Pulmonary tuberculosis	7	58	25	57	32	57
Extrapulmonary tuberculosis	2	17	5	11	7	13
Shingles	1	8	3	7	4	7
Total	**12**	**100**	**44**	**100**	**56**	**100**

Table 3. Antifungal drugs used in main hospitals of Bukavu, South Kivu (DRC).

Antifungal drug	At HGRP		At HPGRB		All patients	
	n	%	n	%	n	%
Amphotericin B IV	1	8	5	11	6	11
Fluconazole IV and oral	11	92	35	80	46	82
Amphotericin B IV and Fluconazole	0	0	4	9	4	7
Total	**12**	**100**	**44**	**100**	**56**	**100**

infected persons. This prevalence of 11% is similar with the results of others studies in central Africa from Cameroon and Burundi (Mbuagbaw et al., 2006; Swinne et al., 1999). In our setting, all CM patients were HIV-infected, and there was a female predominance (*sex ratio* F: M = 2:1). But, in others African studies, more of male predominance with some sporadic cases of *Cryptococcus neoformans* infection occurring among HIV-negative patients was reported (Mbuagbaw et al., 2006; Eholié et al., 1997; Soumaré et al., 2005). Like in others sub-Saharan African studies on cryptococcocal meningitis, clinical features were variable; however, meningismus and weight loss were universally present. Concurrent TB was also commonly thought to be present in 70%. This high rate association of *Cryptococcus* and TB, > 50% was previously reported once again in Bukavu City in 2006 (Masimango et al., 2006). HIV/AIDS remains the only cause of cryptococcocal meningitis in the eastern DRC. The diagnosis of *C. neoformans* can be improved with the specific cryptococcocal antigen (CrAg) test. The CrAg latex agglutination test is not available; however, the new CrAg lateral flow immunochromatographic assay (LFA) distick test (Immy, Inc. Norman, Oklahoma, USA) at US$2 per test would be revolutionary for diagnostics in resource-limited settings. The medical care of this severe AIDS-related infection needs also to be optimized in this region where a high rate of cryptococcocal mortality occurs in routine care.

REFERENCES

Eholié SP, Ngbocho L, Bissagnéné E, Coulibaly M, Ehni E, Kra O (1997). Mycoses profondes au cours du Sida à Abidjan (Côte d'Ivoire). Bull. Soc. Path. Ex. 90:307-311.

Masimango J, Katchunga P, Kashongwe Z (2006). Les facteurs de pronostic de la cryptococcose neuroméningée à l'hôpital provincial de Bukavu:à propos de 38 patients. Ann. Int. Med. 3-7.

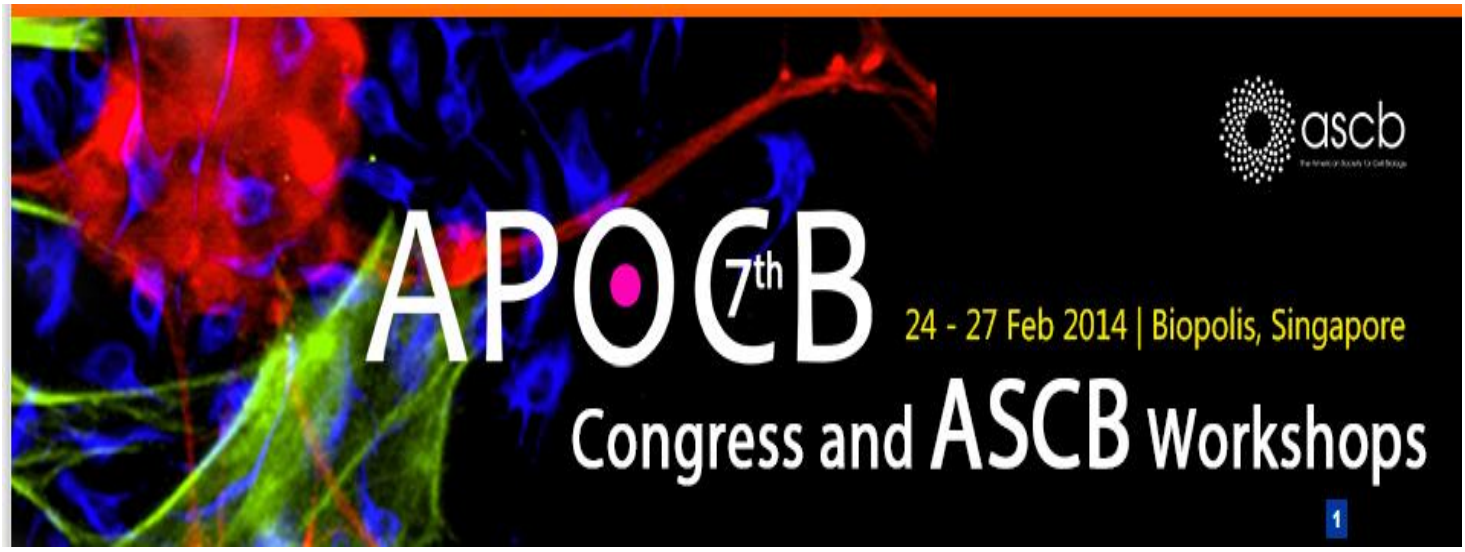
Mbuagbaw JN, Biholong, Njamnshi AK (2006). La cryptococcose neuroméningée et l'infection au VIH dans le service de médecine du centre hospitalier et universitaire de Yaoundé, Cameroun. Afri. J. Neurol. Sci. 25:13-20.

Soumare M, Seydi M, Ndour CT, Dieng Y, Diouf DBM (2005). Aspects actuels de la cryptococcose neuroméningée à Dakar, Med. Trop. 65:559-562.

Swinne D, Deppner M, Laroche R, Floch JJ, Kadende P (1999). Short communication: isolation of *Cryptococcus neoformans* from houses of AIDS associated cryptococcosis patients in Bujumbura (Burundi). AIDS. 3:389-390.

UPCOMING CONFERENCES

7th Asia-Pacific Organization for Cell Biology Congress, Biopolis, Singapore, 24 February 2014



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February 2014
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