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

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Full Length Research Paper

Cultivation and nutritional studies of an edible mushroom from North Brazil

Tamiris Rio Branco da Fonseca, Taciana de Amorim Silva, Mircella Marialva Alecrim, Raimundo Felipe da Cruz Filho and Maria Francisca Simas Teixeira*

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The vertical mycelial growth to investigate the feasibility of *Pleurotus ostreatoroseus* DPUA 1720 production in lignocellulosic Amazonic residues was evaluated. Mycelial development was carried out in cupuaçu exocarp (*Theobroma grandiflorum* Willd Former Spreng Schum), açai seed (*Euterpe oleracea*) and sawdust as substrates. Each residue was supplemented with rice bran, crown and pineapple peel. The average speed of mycelial growth was determined using three replicates for 15 days at 25°C in the absence and presence of light and mycelial vigor and density were evaluated. Five replicates of the selected substrate were used in mushroom production. Vigorous mycelium and strongly dense growth were observed in cupuaçu exocarp treatment supplemented with rice bran. The biological efficiency, production rate and productivity were 22.90, 54.33 and 3.55%, respectively in this substrate. The basidiomata showed low levels of minerals and fat and can be considered as a source of protein (23.53%) and fiber (12.79%).

Key words: basidiomata, edible mushroom, agro-industrial wastes.

INTRODUCTION

The importance of edible mushrooms has increased in recent years because of their gastronomic value, nutritional potential, medicinal properties and ability to degrade and recycle agro-industrial residues (Bonatti et al., 2004; Cheung and Cheung, 2005; Furlani and Godoy, 2005; Pedra et al., 2009).

Pleurotus is an important mushroom genus known for its high content of proteins, carbohydrates, minerals (calcium, phosphorus and iron) and vitamins (thiamine, riboflavin and niacin), as well as low fat content. These mushrooms have the ability to colonize and degrade a

wide variety of lignocellulosic wastes with relatively short cycle (Justo et al., 1998; Manzi et al., 1999; Eira, 2004; Bonatti et al., 2004; Shashirekha et al., 2005; Pedra and Marino, 2006; Toro et al., 2006; Pedra et al., 2009; Menolli Junior et al., 2010; Oमारini et al., 2010).

Among the edible *Pleurotus* genus, *Pleurotus ostreatoroseus* produces bioactive compounds with reducing action of triglycerides in the human body besides the meaty pink color basidiomata have good taste (Nascimento et al., 2008). This species has a worldwide distribution and can be found in tropical or

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Table 1. Substrates used to evaluate the mycelial growth: (A) Basal substrate-açaí seed (AS); sawdust (SAW); cupuaçu exocarp (CE) and (B) supplementation substrate- pineapple peel (PP); pineapple crown (PC); rice bran (RB).

Experiment	(A) Basal substrate	(%)	(B) Supplementation substrate	(%)
1	AS	80	PP	20
2	AS	80	PC	20
3	SAW	80	PP	20
4	SAW	80	PC	20
5	CE	80	RB	20

subtropical forests (Guerrero and Homrich, 1999; Putzke, 2002; Rosado et al., 2002).

This mushroom has been produced *in vitro* in several agro-industrial substrates as castor plant peel, rice straw, sugarcane bagasse, cotton waste, soybean straw, corn cob, elephant grass, oat, rye grass, sunflower seed, eucalyptus sawdust, stillage and privet (Rosado et al., 2002; Donini et al., 2005; Bernardi et al., 2007; Minotto et al., 2008; Nascimento et al., 2008; Aguiar et al., 2010; Reis et al., 2010).

The cultivation of edible mushrooms in agro-industrial residues has been shown as an alternative to better utilization of the organic matter. Usually at the end of cycle the biomass obtained can be used as food due to its high nutritional value. These residues associated with mycelium also have a great potential for use as fodder animal and as fertilizer in agriculture (Bonatti et al., 2003; Shibata and Demiate, 2003).

The Amazon rainforest has an incalculable wealth diversity of micro-organisms, water, ores, plants and animals species. It is also a source of a large organic residues volume that can be used as substrate for the growth of fungi by fermentation processes to produce biomass with biological activity. Based on the availability of various agro-industrial residues from local logging and amazonic fruit production, the aim of this study was to evaluate the use of agro-industrial wastes in vertical mycelial growth of the edible mushroom *P. ostreatoroseus* DPUA 1720, select a mixture of substrates for the mushroom production and verify their nutritional value and microbiological quality.

MATERIAL AND METHODS

Organism and culturing conditions

P. ostreatoroseus DPUA 1720 was obtained from Culture Collection DPUA/Federal University of Amazonas (UFAM). The culture was maintained in Potato Dextrose Agar+0.5% Yeast Extract (w/v) (PDA+YE) under refrigeration (4°C). The matrix culture was prepared in the same medium at 25°C for 8 days in absence of light.

Measurements of vertical mycelial growth

P. ostreatoroseus was cultivated in the following substrates:

cupuaçu exocarp [*Theobroma glandiflorum* Willd Ex-Spreng Schum (CE)], açaí seed [*Euterpe oleracea* (AS)] and sawdust (SAW) [80% (w/v)] supplemented with rice bran [*Oryza sativa* (RB)], pineapple crown [*Ananas* sp. (PC)] and pineapple peel [*Ananas* sp. (PP)] [20% (w/v)] on dry basis, respectively (Table 1) to determinate the vertical micelial growth. The substrates were treated with Pury Vitta® (0.96% p/p active chlorine) according to the manufacturer's recommendations. Excess water was drained and the medium adjusted to 60% moisture and pH 6.5.

The formulations made with the wastes (Table 1) were placed in test tubes (200 x 25 mm) and were sterilized at 121°C for 60 min in three consecutive days. After cooling, three discs (8 mm of diameter) from the matrix culture were inoculated on the substrates surface. Millimeter tapes of 150 mm were placed in three equal distant points of the test tube to follow the growth of mycelium. The cultures were maintained at 25°C, 60% humidity, in absence (experiment 1) and presence of light (experiment 2). The vertical mycelial growth was measured (mm) every 24 h, for 15 days. All the experiments were performed with three replicates.

The mycelial vigor was assessed according to the following classification: weakly dense, moderately dense or strongly dense (Marino et al., 2008). Daily mycelial growth average speed (VMC) was calculated in cm/day according to Equation 1 (Israel, 2005; Palheta et al., 2011).

$$VMC = \frac{V_f - V_i}{T_f} \quad (1)$$

Onde: VMC = Daily mycelial growth average speed; V_f = Final measure of micelial growth (cm); V_i = Initial measure of micelia growth (cm); T_f = Final time (days).

Production of *P. ostreatoroseus* in agro-residues

Spawn production

The spawn was prepared according to Rollan (2003) using wheat grains. The grains were washed, pre-cooked for 15 min and treated with 0.3% calcium carbonate (w/v, dry basis). In flasks of glass (1000 mL), with screw cap containing a central hole capped with a cotton plug, the grains were included and sterilized at 121°C for 60 min. After cooling, 12 discs (8 cm of diameter) from the matrix culture were inoculated into the surface of the substrate. Inoculated flasks were maintained at 25°C in absence of light until the completion of growth of mycelium on substrate.

P. ostreatoroseus solid fermentation

The production of *P. ostreatoroseus* was made in cupuaçu exocarp supplemented with rice bran (CE+RB). The residues on dry weight basis were mixed in ratio 4:1 (800:200, w/w) and treated with 0.3%

calcium carbonate (w/v), pH 6.5. The substrate was distributed in polypropylene bags and sterilized at 121°C for 60 min during three consecutive days. After cooling, the spawn were inoculated in the substrate and a total of five replicates were made.

The incubation was carried out in two cycles. The first one at 25°C, 60% moisture and absence of light until full mycelial colonization of the substrate and the second at 15°C, for 24 h to induce primordia and at 25°C, 90% moisture to basidiomata formation.

During the growing cycle, the cultures were submitted to automatic control of temperature, lightening (12 h a day), moisture and air exchange. In this process the formation and development of the mushrooms and total time of cultivation were evaluated. The basidiomata were collected, weighed and dehydrated at 40°C in a forced air circulation oven. Four parameters of production performance were analyzed: biological efficiency (EB) (Equation 2), productivity (P) (Equation III), production rate (TP) (Equation IV) and loss of organic matter determination (PMO) (Equation V) (Dias et al., 2003;Oliveira et al., 2007; Holtz et al., 2009):

$$\text{Biological efficiency (EB)} = \frac{\text{mushroom mass (wet basis)}}{\text{substrate mass (dry basis)}} \times 100 \quad (2)$$

$$\text{Productivity (P)} = \frac{\text{mushroom mass (dry basis)}}{\text{substrate mass (dry basis)}} \times 100 \quad (3)$$

$$\text{Production rate (TP)} = \frac{\text{biological efficiency}}{\text{total days of cultivation}} \times 100 \quad (4)$$

$$\text{Loss of organic matter (PMO)} = \frac{\text{residual substrate mass (dry basis)}}{\text{initial substrate mass (dry basis)}} \times 100 \quad (5)$$

Proximal composition of substrates and *P. ostreatoroseus*

The dehydrated residues and basidiomata were analyzed for moisture level, protein, ash, fat, carbohydrates, fiber and calories. The protein content was calculated using a correction factor of 4.38 to basidiomata and 6.25 to substrates (AOAC, 1997; Furlani and Godoy, 2005; Silva et al., 2007; Pauli, 2010).

Minerals analysis of *P. ostreatoroseus*

Minerals analysis was performed according to Embrapa methods. The samples were dried in a forced air circulation oven at 40 °C and then submitted to acid digestion in HNO₃ + HCl O₄ (3:1 ratio). Phosphorus content was determined by Ultraviolet-visible spectroscopy. Calcium, magnesium, potassium, copper, iron, manganese and zinc contents were determined by atomic absorption spectrophotometry (AAS). All analyzes were performed in triplicate. The amounts of macrominerals (Ca, P, Mg and K) were calculated in g.kg⁻¹ and trace elements (Fe, Cu, Mn, and Zn) in mg.kg⁻¹.

Amino acid analysis of *P. ostreatoroseus* biomass

The amounts of amino acids analysis were performed by high performance liquid chromatography (HPLC). The samples were submitted to hydrolysis in 6N hydrochloric acid (HCl) followed by derivatization of amino acids with phenylisothiocyanate (PITC), and separation of the phenylthiocarbonyl derivative amino acids in reversed phase column with UV (Ultraviolet) detection at 254 nm.

The quantification was performed by multilevel internal calibration with α-aminobutyric acid (AABA) as internal standard for total amino acids. The determination of tryptophan was performed after hydrolysis with pronase enzyme and color reaction with p-dimethylaminobenzaldehyde (DAB) according to Spies (1967).

Microbiological analysis of *P. ostreatoroseus* dehydrated basidiomata

The health and hygiene conditions of *P. ostreatoroseus* biomass were made according to Brazilian legislation (Brasil, 2001). Analysis of moulds and yeasts were also made although it is not required by the same legislation (WHO, 1998).

In microbiological analyses, 25 g of the dehydrated basidiomata were mixed with 225 mL of peptone water. From this solution, successive dilutions were prepared in tube tests containing 9 mL of 0,1 % peptone water (w/v) until 10⁻³ dilution. Volumes of 100 and 200 µL were removed from 10⁻¹ to 10⁻³ dilutions to determinate moulds and yeasts, total and thermotolerant coliforms, *Salmonella* sp. and *Staphylococcus aureus*.

Moulds and yeasts analysis

From each dilution made, a volume of 200 µL was spread in the surface of Rose Bengal agar with 0.001 % chloramphenicol (w/v). The Petri dishes were incubated at 25°C for seven days. All the experiments were made in triplicates and the results were expressed as colony forming units per gram product (CFU/g) (Silva et al., 2007).

Total and thermotolerant coliforms, *Salmonella* sp. and *Staphylococcus aureus* determination

Most Probable Number test (MPN.g⁻¹) of total and thermotolerant coliforms and *Salmonella* sp. were made removing a volume of 1000 µL to 3 test tubes containing 9 mL of Brila broth (Himedia®, Mumbai-India) with reversed Durham tubes. The tubes were incubated at 37°C for 24-48 h. From the positive results (gas formation), confirmation tests were made. The confirmation for total coliforms was made in Brila broth (Himedia®, Mumbai-India) at 35°C for 24-48 h. Thermotolerant coliforms confirmation was made in *Escherichia coli* broth (EC) (Himedia®, Mumbai-India) at 45°C for 24 h. The values of NMP.g⁻¹ were calculated according the methodology of Silva et al. (2007).

Salmonella sp. test were made from the test tubes with gas formation and maintained at 35 °C. An aliquot was removed and inoculated in Bright Green agar (BG) (Himedia®, Mumbai-India) at 35°C for 24 h. The suspect colonies were tested by biochemical identification of *Salmonella* (Silva et al., 2007).

The quantification of coagulase positive Staphylococci were made from dilutions 10⁻¹ to 10⁻³. A volume of 100 µL were removed and inoculated in 15 ml of Mannitol agar melted and then cooled until 45°C. After mixture and solidification of the medium, the dishes were incubated at 37°C. The measure was made after 24 to 48 h. The results were considered positive by the color change varying from red to yellow and to confirm coagulase positive, three colonies that promoted the color change, were selected with other three colonies atypical to the same test. These colonies were transferred, separately, to 2mL of Brain Heart Infusion broth (BHI) and maintained at 37°C. After 24 h, 300 µL of fermented BHI were transferred to rabbit plasma and incubated at 37°C for 6 h. The positive result was determined by the presence of clot (Reis, 2010).

Statistical analysis

All experiments were submitted to descriptive analysis (tables,

Table 2. Mycelial growth average speed (average) and mycelial vigor of *P. ostreatoroseus* DPUA 1720 cultivated on agrowastes in the presence and absence of light.

Treatment	Presence of light		Absence of light	
	VMC (cm/day)	Vigor	VMC (cm/day)	Vigor
AS + PP	0.27 ± 0.06 ^{ef}	2	0.41 ± 0.04 ^{bcd}	2
AS + PC	0.52 ± 0.05 ^b	2	0.75 ± 0.02 ^a	2
SAW+ PP	0.25 ± 0.02 ^f	1	0.37 ± 0.001 ^{de}	1
SAW + PC	0.33 ± 0.03 ^{def}	1	0.50 ± 0.06 ^{bcd}	1
CE + RB	0.38 ± 0.02 ^{cde}	3	0.41 ± 0.07 ^{bcd}	3

AS+PP= açai seed+pineapple peel; AS+PC= açai seed+pineapple crown; SAW+PP= sawdust+pineapple peel; SAW+PC= sawdust+pineapple crown; CE+RB= cupuaçu exocarp+rice bran. Means followed by same letters do not differ between them by Tukey test ($p \leq 5\%$). 1, weakly dense; 2, moderately dense and; 3, strongly dense.

graphics and frequency distribution), variance analysis and the means were compared by Tukey test (5% of significance) using Minitab software, version 16.0.

RESULTS AND DISCUSSION

Agro-industrial wastes are raw materials useful to biotransformation by micro-organisms to develop products or compounds with various optional uses or applicability in different sectors of the economy (Ezejiofor et al., 2012). Table 2 presents the results of vertical mycelial growth average speed (VMC) and mycelial vigor of *P. ostreatoroseus* DPUA 1720 in AS+PP, AS+PC, SAW+PP, SAW+PC, CE+RB. The data demonstrated the influence of light for mycelial growth. In açai seed with pineapple crown (AS+PC) mycelial developed was significant. According to the following classification: weakly dense, moderately dense, and strongly dense, the mycelial vigor was moderately dense in presence and absence of light.

In cupuaçu exocarp with rice bran (CE+RB) the mycelial vigor was strongly dense in both cultivation conditions. Similar data were obtained by Palheta et al. (2011) with *P. florida* and *P. ostreatus* also cultivated in CE+RB (20% w/w). The fruit exocarps or peels used as substrate or supplement were more efficient to *P. ostreatoroseus* growing because they proportioned satisfactory mycelial vigor. The growing ability of a fungus species, its reproduction and basidiomata development in lignocellulosic substrates is associated to mycelial vigor and the capacity of activating physiological mechanisms during the developing cycle (Albuquerque et al., 2012).

The mycelial development resulting of the experiments probably is not only associated with mushroom cultivation conditions but also to other interfering factors as high concentrations of CO₂ that compromise the enzyme activity. The substrate used as supplement can alter the medium composition. The size of the particles also can

be difficult, the gas changes and retard the growing of the apical hyphae modifying the mycelial speed formation in the bottom of substrate (Yang et al., 2015).

Some factors affect mycelial growth in mushroom cultivation as the culture media, temperature, carbon and nitrogen sources, availability of nutrients and genetic potential (Hoa and Wang, 2015). In this study, *P. ostreatoroseus* expressed distinct values of mycelial vertical speed and similar values of mycelial vigor in the presence and absence of light. The higher-level mycelial biomass was in the cultivations prepared with barks and seeds from Amazon fruit. Similar results were presented by Rivas et al. (2010) evaluating parameters that could confirm the viability of pectinolytic (banana peel and skin of passion fruit) and lignocellulosic (sawdust) substrates in *Pleurotus* spp. cultivation. The authors only confirmed the viability of pectinolytic substrates to mushroom cultivation.

Marino et al. (2008) confirmed that coconut bark sawdust supplemented with wheat bran and rice promoted growing and mycelial vigor of three *Pleurotus ostreatus* strains. Bernardi et al. (2007) used black oat supplemented with 20% wheat bran and observed an expressive colonization of the substrate by *Pleurotus ostreatoroseus*. It probably happened due the relation between carbon and nitrogen sources.

Rice bran was the substrate that presented the highest contents of protein and fat (17.37 and 19.41%, respectively). Pineapple crown, cupuaçu exocarp, pineapple peel, açai seed and sawdust presented protein contents of 16.14, 12.42, 7.99, 7.85, 5.44%, respectively (Table 3).

Fiber content was higher in sawdust (63.53%) and pineapple crown (28.36%). The others substrates presented contents between 0.37 to 9.2%. Ash content was 9.14, 4.53, 3.96 and 3.45% in rice bran, cupuaçu exocarp, pineapple crown and pineapple peel, respectively. The higher values of carbohydrates were determined in açai seed (85.69%), cupuaçu exocarp (71.09%) and pineapple peel (67.96%). Total energy was 415.25 kcal in rice bran and 391.18 kcal in açai seed. In the other substrates, these values were between 134.03 and 351.85 kcal. Different values of centesimal composition were presented in other studies with the same substrates (Costa et al., 2007; Sales-Campos et al., 2010; Jafarpour et al., 2010; Sousa et al., 2011).

The production of *P. ostreatoroseus* in CE+RB 20% (w/w) presented an average of total myceliation, primordia formation and total cultivation in 15.2; 4.2 and 42.2 days, respectively (Table 4). Vega et al. (2006) cited that the total myceliation and primordia formation of *P. djamor* occurred in 13 to 20 days and 42 to 51 days.

P. florida cultivated in different agrowastes presented total substrate myceliation in 21 days, primordial formation in 4 days and total cultivation time in 30 days. In other study, the cultivation of *P. florida* in cotton residue supplemented of 5% (w/v) rice bran presented

Table 3. Average proximal composition of the agro-industrial wastes used in the solid state fermentation.

Parameter	Sawdust	Açai seed	Cupuaçu exocarp	Pineapple crown	Pineapple peel	Rice bran
Humidity*	3.42	0.79	9.61	1.56	9.93	9.24
Ash*	1.33	1.68	4.53	3.96	3.45	9.14
Nitrogen*	0.87	1.26	1.99	2.58	1.28	2.78
Protein* (Nx6.25)	5.44	7.85	1.42	16.14	7.99	17.37
Fat*	1.43	1.89	1.98	2.28	1.47	19.41
Fiber*	63.53	2.1	0.37	28.36	9.2	2.07
Carbohydrate*	24.85	85.69	71.09	47.7	67.96	42.76
Energy (kcal)	134.03	391.18	351.85	275.88	317.01	415.25

*Percent (%).

Table 4. Analyzed parameters during *P. ostreatoroseus* produced in cupuaçu exocarp supplemented with rice bran.

Parameter	Average
Myceliation (days)	15.2 ± 1.3
Primordia formation (days)	4.2 ± 0.84
Total time of cultivation (days)	42.2 ± 2.77
Biological efficiency (%)	22.90 ± 2.27
Production rate (%)	54.33 ± 4.95
Productivity (%)	3.55 ± 0.61
Organic matter loss (%)	37.68 ± 1.39

Table 5. Proximal analysis of *P. ostreatoroseus* basidiomata produced in cupuaçu exocarp supplemented with rice bran.

Parameters	%
Humidity	7.15 ± 0.01
Protein	23.53 ± 0.13
Fat	3.08 ± 0.35
Ash	6.49 ± 0.01
Fiber	12.79 ± 0.09
Carbohydrates	46.98 ± 0.57

mycelial growth in 20 days, total cultivation in 43.4 days. *P. ostreatoroseus* also cultivated in the same residue presented mycelial growth in 20 days, body fruiting bodies formation in 11 days and total cultivation time in 35.6 to 36.8 days (Reis et al., 2010; Figueiró and Gracioli, 2011).

The biological efficiency (EB), production rate (TP) and productivity (P) of *P. ostreatoroseus* DPUA 1720 were 22.90, 54.33 and 3.55% in CE+RB (800 g: 200 g), 60% humidity in dry basis, respectively. Close values were obtained by Oliveira et al. (2007) using peanut bark to produce *P. pulmonarius*. The biological efficiency was around 23% and productivity was 4.58%.

P. ostreatoroseus produced in cotton residue with or without supplementation of 5 % rice bran showed biological efficiency of 104% and 92.5 %, respectively (Reis et al., 2010). Sales-Campos et al. (2010) presented high values of biological efficiency to *P. ostreatus* (64.6% and 125.6%) using pejobaye trunk and balsa wood sawdust as substrates. Low values of biological efficiency can be explained by the organism genetics, culture conditions, substrates composition and its proportion used in the process. Besides, the biological efficiency can interfere in environmental factors as temperature, humidity, luminosity and pH (Oliveira et al., 2007).

The degraded organic material percentage in CE+RB was 37.68% in cultures of *P. ostreatoroseus* DPUA 1720

(Table 4). In Holtz et al. (2009) only 24.10% of the organic matter in cotton spinning residue was degraded after *P. ostreatus* cultivation, however, PMO was 59.91 to 71.83% and 53.58 to 58.75% when the cultivation was made in balsa wood sawdust and pejobaye trunk. *Pleurotus* spp. degradation of passion fruit peel, banana peel and sawdust were 16.63%, 18.59% and 39.79%, respectively (Rivas et al., 2010; Sales-Campos et al., 2010).

Table 5 shows proximate analysis of *P. ostreatoroseus* DPUA 1720 basidiomata. Protein content was 23.53%. This values is according to others recorded to *Pleurotus* species (10.5 to 30.4%) (Furlani and Godoy, 2005). Shimeji mushroom (*P. ostreatus*) commercialized in the city of Campinas presented protein content of 22.22%. *P. ostreatus* and two strains of *P. sajor-caju* cultivated in elephant grass showed protein content of 22.59, 29.24 and 25.51%, respectively. These values are close to the ones obtained by *P. ostreatoroseus* DPUA 1720 (Bernardi et al., 2009; Furlani and Godoy, 2007).

Young and mature mushrooms basidiomata from south of Nigeria presented protein content between 5.1 and 34.1%. Young *P. florida* and *Psathyrella atroumbonata* protein content (15.3 and 18.5%, respectively) were higher than the mature ones (Gbolagade et al., 2006). In Rampinelli et al. (2010) study were determined the proximate composition of two fluxos of *P. djamor*. The

Table 6. Macrominerals and trace elements of *P. ostreatoroseus* produced in cupuaçu exocarp supplemented with rice bran.

Macromineral	(g kg ⁻¹)	Trace element	(mg.Kg ⁻¹)
P	10.39±0.005 ^b	Na	30.85±0.01 ^c
K	24.19±0.006 ^a	Cu	12.47±0.01 ^e
Ca	0.21±0.005 ^d	Fe	72.34±0.01 ^b
Mg	1.46±0.005 ^c	Mn	13.17±0.01 ^d
		Zn	78.06±0.01 ^a

cultivation in banana tree straw presented protein content of 20.5 and 19.8% and fat of 1.12 and 1.09%. Other species like *P. ostreatus* (0.88%), *P. sajor-caju* (0.30 and 0.54%) and *P. florida* (0.9 and 1.2%) also showed low content of fat.

P. ostreatus isolated from Amazon presented 3.08% of fat. This value is considered according to some dry basis mushrooms biomass (1.1 and 8.3%) and close to Shimeji mushrooms (*P. ostreatus*) commercialized in São Paulo (Gbolagade et al., 2006; Furlani and Godoy, 2007; Bernardi et al., 2009). Ash and crude fiber of *P. ostreatoroseus* DPUA 1720 was 6.49 and 12.79%, respectively (Table 5). Mshandete and Cuff (2007) obtained 6.1% of ash and 11% of fiber in *P. flabellatus* basidiomata cultivated on sisal waste. In the same study, *P. djamor* cultivated in banana tree straw presented 6.34% of ash and 12.69% of crude fiber. *P. ostreatus* BF24 presented 18.25% of crude fiber and *P. sajor-caju* PSC96/03 and *P. sajor-caju* PSC01/06 presented 10.32 and 15.34% of crude fiber, respectively (Bernardi et al., 2009; Rampinelli et al., 2010).

Carbohydrates (46.98%) were the main component in *P. ostreatoroseus* DPUA 1720 basidiomata. Other mushrooms like *P. ostreatus*, *P. flabellatus*, *P. sajor-caju* and *P. djamor* presented an average of 25.69 to 60% of carbohydrates. These differences are according to species and cultivation substrate. Total energy of *P. ostreatoroseus* DPUA 1720 presented 309.7 kcal while in *P. flabellatus* and *Volvariella volvaceae* were 302 and 305 kcal, respectively (Mshandete and Cuff, 2007; Bernardi et al., 2009; Rampinelli et al., 2010).

The nutritional characteristics of mushroom species related in different publications can be associated to some conditions as climate, growth condition, regional characteristics and type of management. In *P. ostreatoroseus* mushrooms the macrominerals K and P were significant while Mg and Ca were determined in minor amounts. Among the trace elements, Zn and Fe had the highest concentrations and Na, Mn and Cu were present in small quantities (Table 6). The mineral elements are essential for many metabolic processes and play an important biological role on the function and cellular structure (Masamba and Kazombo-Mwale, 2010; Soetan et al., 2010; Osredkar and Sustar, 2011; Mallikarjuna et al., 2013). *P. flabellatus* presented 16.2

Table 7. Amino acids concentration in *P. ostreatoroseus* DPUA 1720 basidiomata.

Amino acids (g/100 g of basidiomata)	Values
Lisine (Lys)	1.298
Metionine (Met)	0.298
Valine (Val)	1.134
Triptofano (Trp)	0.330
Treonine (Thr)	0.937
Isoleucine (Ile)	0.751
Leucine (Leu)	1.304
Fenilalanina (Fen)	0.805
Histidine (Hys)*	0.379
Arginine (Arg)*	1.891
Tirosine (Tyr)	0.743
Aspartato (Asp)	2.061
Serine (Ser)	1.466
Glicine (Gly)	1.037
Proline (Pro)	0.772
Cisteine (Cys)	0.040
Glutamato (Glu)	3.592
Alanine (Ala)	1.432
Total	20.27 g

*Conditionally essential amino acids.

and 15.37 g.kg⁻¹ of phosphorus and potassium, respectively. *P. ostreatoroseus* from São Paulo presented 91.0, 25591, 51.5 and 93.4 mg.kg⁻¹ of iron, potassium, copper and manganese, respectively, while *P. eryngii* presented 16.7 and 20.3 mg.kg⁻¹ of copper and manganese (Mshandete and Cuff, 2007; Moura, 2008; Genççelep et al., 2009).

Bender (2004) reports that *P. ostreatoroseus* contains eight essential amino acids. In *P. ostreatoroseus* DPUA 1720 the most abundant were valine, lisine and leucine ranging from 1.134 to 1.304 g/100 g (Table 7). The content of glutamate and aspartate (nonessential aminoacids) were 3.592 and 2.061 g/100 g. Both have important roles as brain stimulatory neurotransmitters and enhancing foodflavor (Rodrigues et al., 2004).

Histidine and alanine values (Table 7) were close to the ones obtained in *Agrocybe chaxingu* (0.30 and 1.03

g/100 g). However, amino acids concentration in *Flammulina velutipes* and *P. ostreatus* cultivated in wheat, cotton and soy were lower (Dundar et al., 2009; Lee et al., 2011).

The microbiological assessment of *P. ostreatoroseus* mycelial biomass showed absence of molds, yeasts, *Salmonella* sp., total and thermotolerant coliforms or *E. coli*, coagulase positive *Staphylococcus*, mesophilic bacteria and *Bacillus cereus*. Therefore, microbiological analysis revealed that the mushroom was within microbial safety standard specifications and can be considered as safe food.

Conclusion

P. ostreatoroseus grew in all substrates, however the higher level mycelial biomass was in cupuaçu exocarp with rice bran (80:20%) in the presence of light. These Amazon residues properties show that they can be used as substrates in edible mushrooms production, which promotes the reduction of environmental contamination and enable the developing of new protein-rich food. *P. ostreatoroseus* has good appearance, texture and flavor with great contents of crude fiber and proteins, low content of fat and the presence of amino acids and minerals.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Occurrence of begomoviruses in cotton-vegetable agro ecosystem in India

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Begomoviruses are the important pathogens of variety of crop plants and are responsible for causing huge economic losses. It is assumed that cotton vegetable agro eco system plays a vital role in the complex etiology of the diseases caused by begomoviruses. Therefore, the present work was planned to study the relationship among begomoviruses infecting cotton, chilli, radish, tomato and papaya. For this purpose, the symptomatic leaves of these plants were collected and were processed for DNA isolation. The amplified coat protein gene of begomoviruses was cloned and sequenced. The phylogenetic analysis showed that begomoviruses infecting cotton are totally different from the begomoviruses infecting other crops. Based on the coat protein gene sequences, the viruses infecting cotton were identified as cotton leaf curl virus, chilli leaf curl virus in chilli, radish leaf curl virus in radish, tomato leaf curl virus in tomato and papaya leaf curl virus in papaya.

Key words: Begomovirus, cotton, vegetables, variability.

INTRODUCTION

During the last two decades, geminiviruses have emerged as devastating pathogens causing huge economic losses and threats in agricultural production. These viruses form the second largest family of plant viruses, the *Geminiviridae*. Based on the genome organization, insect vector, host range and sequence relatedness, earlier the family *Geminiviridae* was subdivided into four genera: *Mastrevirus*, *Curtovirus*, *Topocuvirus* and *Begomovirus* (van Regenmortel et al., 2000; Fauquet and Stanley, 2003). But recently, three new genera *Becurtovirus*, *Eragrovirus* and *Turncurtovirus*

have been created in the family *Geminiviridae* (Varsani et al., 2014). More than 80% of the known geminiviruses are transmitted by whitefly in a semi-persistent circulative manner but not by sap inoculation (Muniyappa et al., 1991) and infect dicotyledonous plants. They belong to genus *Begomovirus* (Rojas et al., 2005). These viruses are the important pathogens of a variety of crops like cotton, grain legumes, cassava and vegetables (tomato, chilli, okra and cucurbits) in the tropical and sub-tropical areas (Varma and Malathi 2003; Kang et al., 2004) and are responsible for causing crop yield losses between 20-

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100% (Sastry and Singh, 1973; Singh et al., 1979; Brown and Bird, 1992; Sivalingam et al., 2007).

Begomoviruses are known to induce distinct types of symptoms of leaf curl, vein yellowing, severe reduction in leaf size, downward curling, crinkling of interveinal areas, interveinal and marginal chlorosis, occasional development of enations on the underside of leaves, purple discoloration of the abaxial surface of leaves, shortening of internodes, development of small branches and reduced fruiting. Therefore, most of the diseases caused by begomoviruses have been collectively described as either leaf curl or yellow leaf curl or yellow vein based on their biological properties and subtle differences in symptoms.

Based on phylogenetic studies and genome arrangement, begomoviruses have been divided broadly into two groups: the Old World (OW) viruses (eastern hemisphere, Europe, Africa, Asia) and the New World (NW) viruses (western hemisphere, the Americas) (Rybicki, 1994; Padidam et al., 1999; Paximadis et al., 1999). Begomovirus genomes have a number of characteristics that distinguish OW and NW viruses. All indigenous NW begomoviruses are bipartite, whereas both bipartite and monopartite begomoviruses are present in the OW. The length of the genome of monopartite begomoviruses is about 2800 nucleotides (Navot et al., 1991) while the bipartite begomoviruses contain two nearly equal-sized DNA molecules of size 2600-2800 nucleotides which are designated as DNA-A and DNA-B.

These genomic components of a given single virus differ in their function and nucleotide sequences, except for the sequence of an intergeneric region of about 200 nucleotides called the common region (Lazarowitz et al., 1992). This region encompasses a stemloop that contains the conserved TAATATTAC nonanucleotide sequence, common to all viruses and this sequence helps in the initiation of rolling circle replication of circular DNA. The component, DNA-A encodes for all viral functions necessary for virus replication and encapsidation of viral DNA (Lazarowitz, 1992). The DNA-B encodes for functions associated with virus movement within the plant and symptom expression (Revington et al., 1989). Most of the bipartite begomoviruses require both DNA components for infection but not the coat protein (Padidam et al., 1995) while the monopartite viruses may require the presence of single stranded DNA molecule known as DNA- β . This DNA- β consists of unrelated sequences half the size of their helper begomoviruses (1370 nucleotides), encoding a single gene known as β C1 which is the major pathogenicity determinant of the complex while for replication, encapsidation and movement in plants and for transmission, it depends upon the helper virus that is DNA-A. In addition to these, DNA alpha satellites have also been found to be associated with DNA-A and DNA- β complex (Mansoor et al., 1999).

Begomoviruses are successfully transmitted by silver

whitefly (*Bemisia tabaci* Gennadius) in a semi-persistent manner, which has been known to be polyphagous with a very wide host range. The appearance of new biotype of vector (B-biotype) has resulted in the emergence of new begomoviruses and its movement into new regions and high fecundity have increased the incidence of begomoviruses around the world (Brown and Bird, 1992).

Considerable variability has been found in virus isolates from different parts of India (Varma and Malathi, 2003). Several factors including evolution of new variants of the viruses, appearance of efficient vectors, weather events, changing cropping systems, movement of infected planting material and introduction of susceptible plant varieties individually, or in combination, have contributed to the emergence of begomovirus problems around the world.

Begomoviruses have wide host range infecting large number of crops like cotton, cucurbits, okra, tomato, chilli belonging to different families like *Malvaceae*, *Solanaceae*, *Brassicaceae* and *Cucurbitaceae* etc. These crops are known to play a very important role in farmer economy. In this region, cotton growing season overlaps with the growing season of vegetables like chilli, tomato, radish, okra etc. Therefore, it is assumed that cotton vegetable agro-ecosystem may play a vital role in the higher incidence and emergence of new begomoviruses. Many workers have studied the variability of begomoviruses on individual crops but very little work has been done in the cotton vegetable agro ecosystem. Considering the occurrence of begomoviruses on these crops and their importance, the present investigations were aimed to study the phylogenetic relationship among begomoviruses infecting cotton, tomato, chilli, radish and papaya growing in the region.

MATERIALS AND METHODS

Sample collection

The infected samples of cotton and three vegetable crops viz., chilli, tomato, radish and one fruit plant papaya showing typical symptoms of begomoviruses like leaf curling, vein thickening, and yellow veins were collected from different locations of Punjab during the year 2010 and 2011.

DNA Isolation

The DNA from these samples was isolated using CTAB method as described by Ghosh et al. (2009) where 850 μ l of CTAB buffer containing PVP and mercaptoethanol was added to 100 mg freshly prepared powder of infected leaves and was incubated for 55 min at 65°C in serological water bath. To remove the proteins, 800 μ l of chloroform: isoamylalcohol (24:1) was added and kept on the Rocker for 30 min. After centrifugation, an equal volume of ice-cold Isopropanol was added to the supernatant to precipitate the DNA which was dissolved in 1X TE.

Rolling Circle Amplification

The isolated DNA was quantified and the poor quality DNA was

amplified by rolling circle amplification using illustra™TempliPhi 100 Amplification kit (GE Healthcare) according to the manufacturer's instructions using 50 ng of DNA as template. These RCA products were quantified on 1% agarose gel containing ethidium bromide (10 mg/ml).

PCR

For the detection of begomoviruses in the collected samples, universal degenerate primers (Wyatt and Brown, 1996) were used. The PCR was performed in a 25 µl reaction mix where 100 ng of the DNA taken as template was amplified using Green GoTaq (Promega, USA). The reaction mix finally consisted of 200 µM each dNTP, forward and reverse primers (20 pmol), 1.5 mM MgCl₂ and 10 X PCR buffer. The PCR cycles set were, initial one cycle at 94°C of 1 min, 52°C of 1:30 min, 72°C of 2 min followed by 35 cycles of denaturation at 94°C (45 s), annealing at 52°C (1 min) and extension at 72°C for 1:30 min; the final extension was given at 72°C for 10 min. The PCR products were analyzed on 1% agarose gel.

Purification of PCR products

For the purification of PCR products, the reaction was run for 50 µl containing same concentrations of the reaction mix as already discussed. The PCR products were purified to remove the primer dimers, extra dNTPs and contaminants present along with the desired amplified product using Wizard SVGel and PCR purification kit (Promega, USA) according to the manufacturer's instructions.

Cloning of amplified product

After confirming the presence of begomoviruses, the amplified coat protein gene of DNA-A was cloned into pTZ57R/T vector using InsTAclone™ PCR Cloning kit by Fermentas, UAB, Luthiana. The transformed cells were plated immediately on pre-warmed LB-amp X-Gal IPTG agar plates which were incubated overnight at 37°C to get the blue white colonies. From the white colonies, the plasmids were isolated and the presence of insert in the isolated plasmids was confirmed by PCR using universal M13 primers (M13F: 5' TGTAACACGACGGCCAGT 3', M13R: 5' AGGAAACAGCTATGACCATG 3'). The amplified products were visualized on 1% agarose gel stained with ethidium bromide (10 mg/ml) under UV light. The isolated plasmids (100 ng/µl) were submitted to Bioserve Sequencing Pvt Ltd, Hyderabad, along with the M13 primers (10 pmol).

Analysis of sequences

The sequences obtained were submitted to GenBank database. The basic local alignment search tool (BLAST) search analysis of nucleotide sequences of virus isolates was done using BLAST with sequences available in GenBank database (www.blast.ncbi.nlm.nih.gov/html). The nucleotide sequences for all the isolates were multiple aligned using *MegAlign* (Lasergene Core Suit, www.dnastar.com) and the neighbor-joining phylogenetic tree was constructed with Clustal W program using *MEGA 5* software (Tamura et al., 2011) with bootstrap value of 1000. The virus sequences used for phylogenetic analysis have been listed in Table 1.

RESULTS AND DISCUSSION

To study the relationship among begomoviruses infecting

cotton and other hosts in Punjab, the infected leaves of cotton showing different kinds of symptoms like vein thickening, leaf curling and enations (Figure 1) were collected from three different locations viz., Muksar, Faridkot and Ludhiana districts of Punjab state (India).

All these symptoms are the characteristics symptoms resembling with the symptoms of cotton leaf curl disease caused by cotton leaf curl virus as has been previously reported by Watkins (1981) and Hameed et al. (1994). Kapur et al. (1994) described the symptoms of disease as thickening of veins, curling and puckering of leaves and formation of enations on the underside of the leaves.

The suspected hosts of begomoviruses belonging to different families and growing during different seasons other than cotton were sampled from Ludhiana (Table 2). The plants showing typical symptoms (Figure 2) characteristically of begomoviruses were collected. These included chilli, tomato, radish, and papaya. The symptoms of leaf curl, reduction in leaf size and internodal distance were observed on chilli plants. The tomato plants also showed typical leaf curl and reduction in leaf size. The only leaf curl types of symptoms were exhibited by radish plants. The papaya plants showed leaf curl, puckering and reduction in leaf size type of symptoms. Kumar et al. (2012) observed the upward curling, crinkling, puckering, reduction of leaf area along with stunting of whole plant in the plants of chilli infected with leaf curl disease. Leaf curl type of symptoms in radish associated with radish leaf curl disease in Northern parts of India has been reported by Singh et al. (2012).

Detection of begomoviruses

After isolating DNA from the collected samples, it was quantified with the help of NanoDrop. To increase the amount of circular DNA of begomoviruses, the isolated DNA was subjected to Rolling Circle Amplification (RCA) which yielded high quantity of DNA (Figure 3). One µl of this high quantity DNA was dissolved in 9µl of double distilled water which was further used for detecting begomoviruses with PCR.

In the symptomatic samples of cotton collected from different cotton growing areas of Punjab, the association of begomovirus was confirmed with PCR using different universal degenerate primers (Wyatt and Brown, 1996). Amplified products were analyzed on 1.5% Agarose gel stained with Ethidium Bromide, prepared in 1x TAE buffer. These primers amplified the coat protein gene of size 575 bp (Figure 4a). All the symptomatic plants showed the amplification of this region. The same samples were also processed for PCR after their RCA (Figure 4b)

It was observed that the PCR after RCA yielded better amplified products. Polymerase chain reaction has been reported as the quickest way of detecting begomoviruses (Kang et al., 2004), that is why it is being used

Table 1. List of begomoviruses used for phylogenetic analysis.

Accession no.	Name
KM065514	Cotton leaf curl Rajasthan virus
KJ959630	Cotton leaf curl Rajasthan virus
HQ158011	Cotton leaf curl virus isolate Sirsa-Haryana
HQ158010	Cotton leaf curl virus isolate Mohanpura-Rajasthan
HM235774	Cotton leaf curl virus isolate Naruana-Punjab
HM037920	Cotton leaf curl virus isolate Sirsa-UC segment
JF502364	Cotton leaf curl Rajasthan virus isolate In:Abohar5:2010
AY765254	Cotton leaf curl Rajasthan virus - [Sirsa:04]
AJ228595	cotton leaf curl virus
X98995	Cotton leaf curl Multan virus-[Faisalabad1]
AJ002459	Cotton leaf curl Multan virus-[Okra]
KJ649706	Chilli leaf curl India virus
FM210475	Chilli leaf curl virus
HM140365	Chilli leaf curl virus-HD [India:New Delhi:Papaya:2007]
FM21047	Chilli leaf curl virus
DQ376037	Papaya leaf curl virus
AY691901	Tomato leaf curl virus
KC222953	Tomato leaf curl virus
GQ139516	Papaya leaf curl virus
JQ411026	Radish leaf curl virus
GU732204	Tomato leaf curl Pakistan virus
GU732203	Radish leaf curl virus isolate IN:Bih:ok09
AJ436992	Papaya leaf curl virus
JX524172	Tomato leaf curl Karnataka virus
DQ343284	Tomato leaf curl virus
AY375241	Tomato leaf curl Karnataka virus-[tomato:Lucknow]
EU604297	Tomato leaf curl Karnataka virus
U38239	Tomato leaf curl Karnataka virus
FM877858	Chilli leaf curl India virus
HQ630856	Papaya leaf curl virus isolate Lucknow
FJ514798	Tomato leaf curl Karnataka virus – Bangalore [India:Punjab:Mentha:2007],
HM134235	Tomato leaf curl Karnataka virus-B1 [India:Haryana:Papaya:2009]
HM140368	Papaya leaf crumple virus-Nirulas [India:New Delhi:Papaya:2007],

**Figure 1.** Different types of symptoms observed on cotton plants. **a)** Vein thickening; **b)** enations; **c)** leaf curling.

Table 2. List of collected samples with their symptoms and locations.

Host plant	Location	Symptoms	Isolate number
Cotton	Muhtsar-Bam	Vein thickening	M1
Cotton	Muhtsar	Vein thickening, enations, curling, stunting	M2
Cotton	Faridkot-Tehna	Vein thickening and enations	F1
Cotton	Ludhiana	Vein thickening and upward curling	L1
Chilli	Ludhiana	Leaf curl, reduction in leaf size and intermodal distance	LC
Radish	Ludhiana	Leaf curl	LR
Tomato	Ludhiana	Leaf curl and reduction in leaf size	LT
Papaya	Ludhiana	Curling and puckering of leaves and reduction in leaf size	LP

**Figure 2.** Symptoms observed on a) chilli, b) tomato c) radish and d) papaya.

worldwide for the preliminary detection of the begomovirus. Khan and Ahmad (2005) also used begomovirus specific primer to detect the presence of cotton leaf curl virus in the infected samples of cotton leaves. However, now a days, Rolling circle amplification (RCA) technique is successfully used to increase the virus concentration which helps in avoiding any false negatives and the increased amount of circular viral DNA gives better results in restriction analysis (Johne et al., 2009; Zaffalon et al., 2012).

Characterization of begomoviruses

The purified PCR products were cloned and multiplied in

JM109 competent cells. The recombinant plasmids were isolated and obtained sequences were submitted to GenBank under accession numbers KM923991 (M1), KM923992 (M2), KM923993 (F1), KM923994 (L1), KM923995 (LC), KM923996 (LR), KM923997 (LT) and KM923998 (LP). The phylogenetic tree (Figure 5) show that the begomoviruses of each host forms a different clade than the other. That means the begomoviruses infecting cotton are totally different from the begomoviruses infecting other hosts viz., chilli, radish, tomato and papaya. Based on the phylogeny, it is clear that the cotton begomoviruses (KM923991, KM923992, KM923993 and KM923994) belong to cotton leaf curl virus group. The accession number KM923995 (isolate

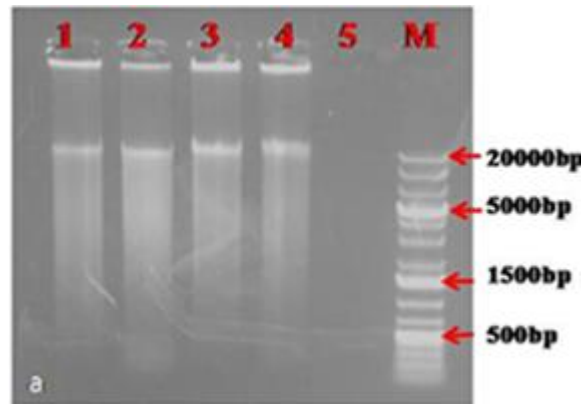


Figure 3. Gel electrophoresis of RCA products (1, 2, 3, 4: RCA products, 5: blank, M: marker).

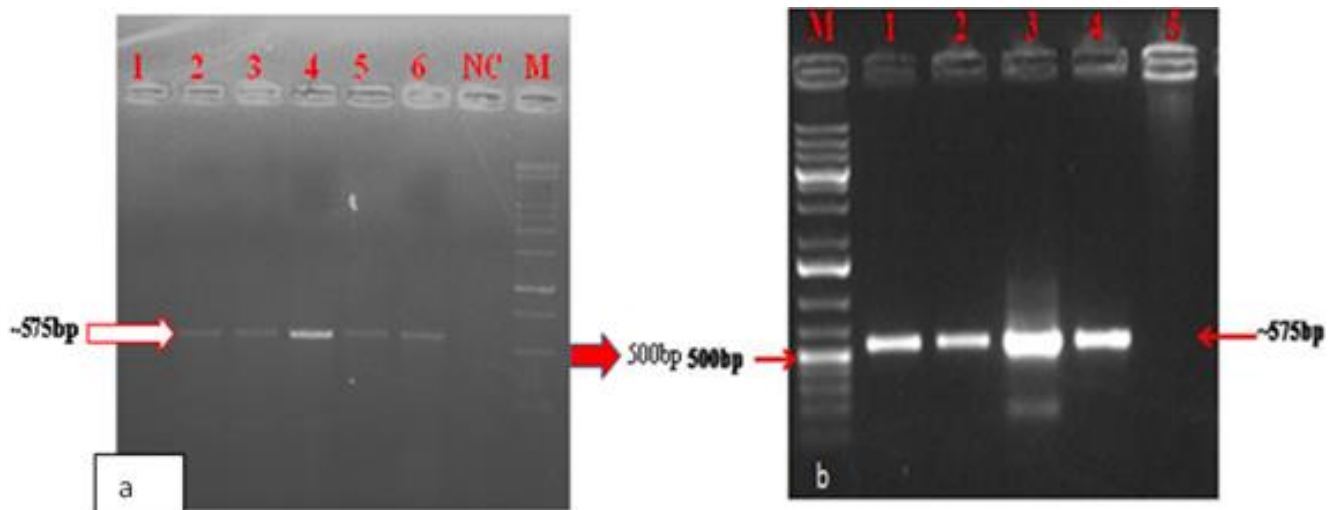


Figure 4. a) Gel electrophoresis of PCR after DNA isolation (1 to 6: infected samples, NC: negative control). b) Gel electrophoresis of PCR after RCA.

from chilli) shared maximum identity with chilli leaf curl virus. The radish begomovirus (KM923996) is closely related to Tomato leaf curl virus (Pakistan), acc no. GU732204. KM923997 from tomato forms a clade with tomato leaf curl virus while KM923998 from papaya was identified as papaya leaf curl virus.

Similar kind of variability in begomoviruses infecting different hosts was reported by Rajagopalan et al. (2012) which stated that begomoviruses infecting cotton are different from the begomoviruses of other hosts. Chattopadhyay et al. (2008) demonstrated that chilli leaf curl disease is caused by a complex consisting of the monopartite chilli leaf curl virus and a DNA- β satellite component. In these studies also, chilli leaf curl virus was found in association with chilli leaf curl disease. Singh et al. (2012) reported a radish leaf curl virus in Northern India which shared maximum nucleotide similarity with

tomato leaf curl virus.

Conclusion

Based on the coat protein gene sequencing, it is concluded that begomoviruses infecting cotton are totally different from the begomoviruses infecting other crops viz., chilli, radish, tomato and papaya which are assumed to play an important role as an alternate hosts of cotton leaf curl virus. Thus, cotton vegetable agro eco system does not play any role in the complex etiology of the diseases caused by begomoviruses.

Conflict of interests

The authors did not declare any conflict of interest.

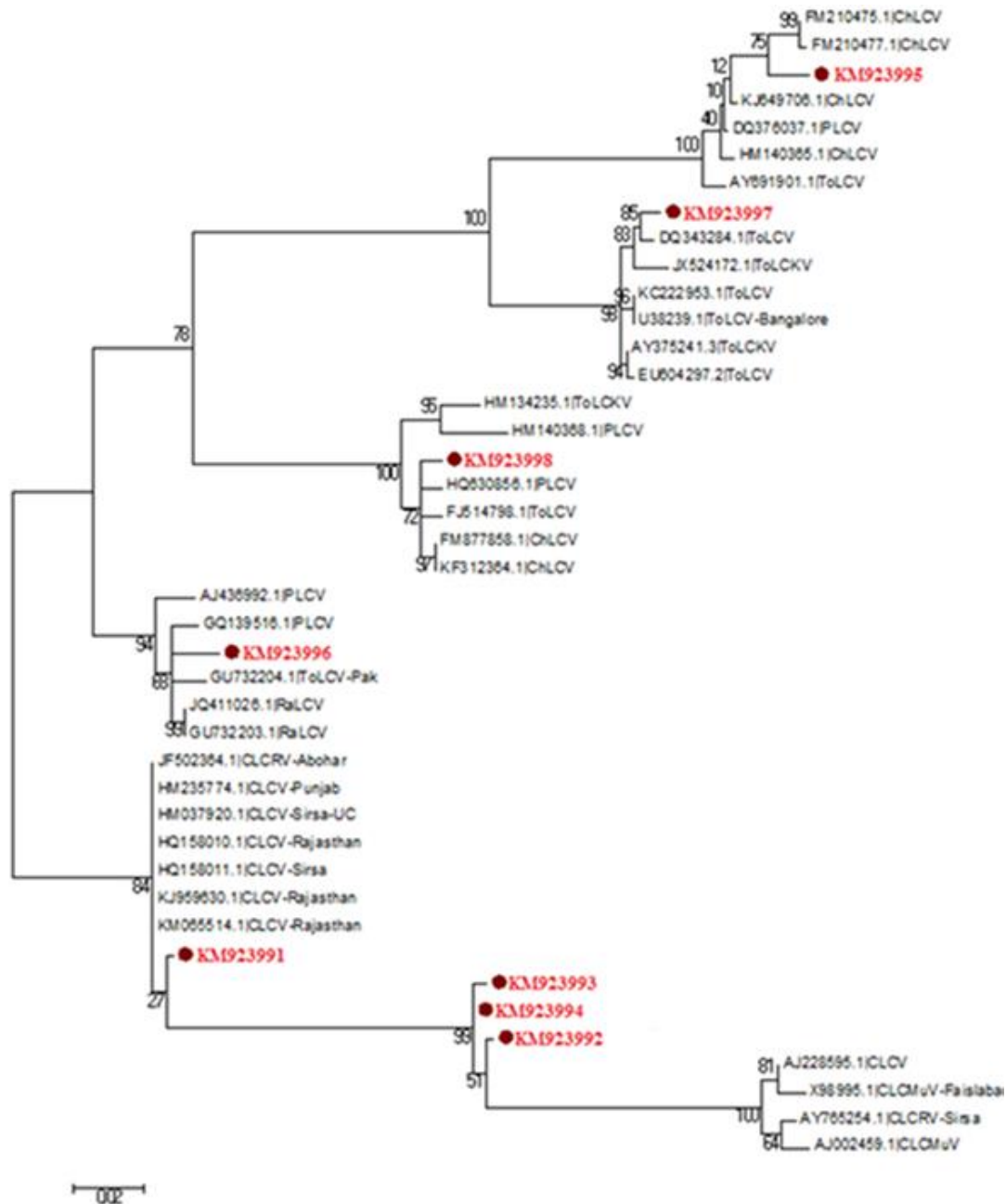


Figure 5. Phylogenetic tree for begomoviruses infecting different hosts.

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Full Length Research Paper

Evaluation of hygienic-sanitary quality of honey from *Apis mellifera* L. obtained in semi-arid region of Piauí, Brazil

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This study aimed to evaluate the quality of honey from *Apis mellifera* L. obtained in Piauí, Brazil. The completely randomized design (CRD) was used in the experiments. Two treatments of honey were prepared: one from beekeepers that use Extraction Units for Bee Products (EUBP) with Best practices for beekeeping (T1), and another one from those which use EUBP without the best practices (T2). Parameters analyzed were: moisture, water activity (aw), pH, acidity, color, detection of *Salmonella* spp., MPN.g⁻¹ of coliforms at 35°C and at 45°C, counting of coagulase-positive *Staphylococcus*, standard counting of mesophilic heterotrophic bacteria and detection of yeast and filamentous fungi. The counting of mesophilic heterotrophic bacteria and yeast and filamentous fungi showed abnormalities (p<0.05) in the counting performed in log₁₀.g⁻¹ with samples of T1 and T2, respectively. There were presence of fungi of various genus and species, especially *Aspergillus* spp. and *Penicillium* spp. The quality of honey from *Apis mellifera* bees from Piauí, Brazil, was satisfactory regarding parameters of moisture, aw, pH and HMF. Neither *Salmonella* spp., nor coliforms, nor coagulase-positive *Staphylococcus* were found. The presence of filamentous fungi in the samples reinforces the need for quality control of honey from Piauí, Brazil.

Key words: Physical chemistry, Hygiene and sanitary quality, Fungi, Best practices for beekeeping.

INTRODUCTION

Honey is one of the oldest food linked to the human history and had always attracted the attention of the man, especially because of their sweetening characteristics (Silva et al., 2004; Bera and Almeida-Muradian, 2007). This product is consumed worldwide because it is considered a natural and energetic sweetener, with predominance of sugars, glucose, fructose, saccharose (70% of carbohydrates) and the water in which the sugars are dissolved (Crane, 1983; Barros and Batista, 2008; Aroucha et al., 2008).

In Brazil, the commercial production of honey is related to the beekeeping, whose history had its beginning with the insertion of the European *Apis mellifera* bees in the State of Rio de Janeiro in 1839. After the development of adequate handling techniques in the 70's, the beekeeping turned out to be intensely practiced in all States of Brazil (Souza, 2004).

Furthermore, due to the high international demand for the product and the favorable exportation prices, the apiculture in Brazil changed from a craft activity focused in the domestic market to an entrepreneurial activity with more elaborate and productive techniques focused in the external market. Data from FAO unveil that Brazil has reached the seventh place in exports of honey, with a quantity of 22 thousand tons and a value of US\$70,879, benefiting all regions of the country (FAO, 2011).

Regionally, the Northeastern production is in ascension. Between 1999 and 2005 it has reached 10.9 thousand tons and achieved the second place, behind of the South region of Brazil, which traditionally occupies the first place and achieved a production of 15.8 thousand tons of honey (IBGE, 2006). Such a fact reflected in 2009, when the Northeastern region was responsible for the production of 14.9 thousand tons of the whole Brazilian production, keeping its second place and approaching the South region, which produced 16.5 thousand tons of honey (IBGE, 2009).

Thus, like the other States of Northeast, Piauí has a high potential for honey production due to its environmental conditions and its melittophilous vegetation, which make of the beekeeping an outstanding activity in the State as well as in the country. It is of note that Piauí was able to insert honey as an important product among the worldwide exportation commodities. In 2005 and 2006, Piauí was the third biggest producer of honey of Brazil and in 2009 it became the fourth biggest producer in the country (Moura et al., 2013; IBGE, 2006, 2009).

This productive scenario must conform to numerous quality criteria and certifications, before its commercialization and exportation, once they are subject to

frauds, adulteration and contamination due to inadequate manipulation (Silva et al., 2008). The microorganisms commonly found in this product are bacteria in its sporulated form, like *Bacillus*, yeasts and fungi, as the ones of the genus *Penicillium*, *Mucor*, *Aspergillus* and *Saccharomyces* (Snowdon and Cliver, 1996; Sodr e et al., 2007).

Due to this, the concern with the quality of the honey produced in Piauí became relevant, as well as the knowledge of the microorganisms that are most used as quality indicators in order to conform to the market demands, especially the foreign market.

It is still a reality in the State of Piauí the existence of beekeepers that are in a craft category and ones that use methods for the control of the quality of the extraction established in some Extraction Units for Bee Products (EUBP). The use of the EUBP favors the security of the product when essential cares are taken in order to obtain a good quality honey. To do this, the EUBP and the implantation of Best Practices for Beekeeping (BPB) result in a quality improvement of the honey produced in the State and this necessity appeared with the arousing of exportations and the demands from the foreign market (Vilela, 2000; Moura et al., 2014).

Thus, to diagnose the quality of the honey of Piauí is important as a way to direct the support activities that will help to develop small and large producers. The aim of this study was to evaluate the quality of the honey of *Apis mellifera* L. bees obtained from beekeepers of Piauí that use the EUBP with the Best Practices for Beekeeping (BPA) and of that ones who use the EUBP without the Best Practices for Beekeeping.

MATERIALS AND METHODS

Initially a survey was conducted to assess the main municipalities regarding production and exportation of honey in Piauí; it was found that the cooperatives that suited the objectives of the study were concentrated in the central South region of the State, where semi arid climate predominate (Brasil, 2009). Inside that region, were randomly selected the cooperatives of the cities of Picos (07° 04' 37" S; 41° 28' 01" W), S mpl cio Mendes (07°51'14" S; 41°54'37" W) and S o Raimundo Nonato (09°00'54" S; 42°41'56" W), to the acquisition of honey samples directly from the beekeepers.

The experimental design was the completely randomized design (CRD), with two treatments (T1 and T2) to the honey acquired from beekeepers, summing 54 samples of honey, with 27 collected for treatment. It was considered as treatments, in the scope of this study, the samples of honey from two groups of beekeepers (manipulators) were: the ones that use EUBP with Best Practices for Beekeeping (T1); and the ones that use EUBP without Best Practices for Beekeeping (T2).

Fifty four (54) samples of honey (27 per treatment) were collected,

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Table 1. Averages and standard deviation of physicochemical analyses of honey of *Apis mellifera* L bees.

Treatment	Physicochemical parameter					
	Moisture	aw	pH	Acidity (meq.kg ⁻¹)	Color (mm)	HMF (mg.kg ⁻¹)
T1	17.8 ^b ±0.55	0.68 ^b ±0.08	3.72 ^a ±0.43	39.3 ^a ±21.34	63.85 ^a ±14.19	18.3 ^a ±15.17
T2	18.2 ^a ±0.96	0.76 ^a ±0.03	3.52 ^a ±0.37	59.1 ^b ±24.24	61.14 ^a ±13.59	16.1 ^a ±3.77
P	0.041	0.0001	0.0719	0.0024	0.4779	0.4735
Maximum Reference Value (Brasil, 2000)	20.0	-	-	50.0	-	60.0

T1, Honey from beekeepers that use the EBUP with Best Practices for Beekeeping. T2, Honey from beekeepers that use the EBUP without Best Practices for Beekeeping. aw, Water activity; meq, milliequivalent. ^aAverages followed by the same letter in the lines and in columns do not differ between themselves by the Tukey's test ($p < 0.05$).

from March to April of 2010, which were aseptically held in sterilized bottles of 350 ml of capacity, and wrapped in a plastic-bag for first-use food. The samples were sent to Laboratory of Microbiological Control of Food of the Food Processing, Research and Studies Center (NUEPPA), of the Centre for Agricultural Sciences, and the Interdisciplinary Laboratory for Advanced Materials (LIMAV), of the Natural Sciences Center, both centers belonging to the Federal University Piau  (UFPI), to perform the analyzes.

The physicochemical analyzes constituted the maturity indicators of the honey: moisture and water activity (aw); indicators of deterioration of the honey: pH, acidity and hydroxymethylfurfural (HMF); and the sensorial feature of color. All the analyzes were performed in triplicate, following methods predetermined by the Brazilian laws (Brasil, 2000). The procedures used were in conformation with the analytical norms of the Association of Official Analytical Chemists (AOAC, 1998). The analyzes of water activity (aw) were performed using the digital model of aw determiner Decagon Pawkit.

In all samples of honey sent to the laboratory, 25 g of honey were removed, weighted aseptically and then added to 225.0 mL of peptone saline 0.1%, in order to obtain an initial dilution of 10^{-1} and, from this dilution, decimal dilutions were prepared until 10^{-3} dilution was reached. The microbiological analyzes of the study were focused on the occurrence of *Salmonella* spp., most probable number of coliforms at 35°C and at 45°C, coagulase-positive *Staphylococcus* count and standard counting of mesophilic aerobic microorganisms, which were based on methodologies described in 62nd Normative Instruction (Brasil, 2003).

The serial dilution in its decimal form as described by Pitt and Hocking (2009) was obeyed for standard counting in dishes of filamentous fungi and yeasts and for the identification of the fungi species. Inocula were aliquots of 0.1 mL per Petri dish, on the surface of the medium of Dichloran Rose Bengal Chloramphenicol (DRBC), in duplicate, in each of dilutions used to the general counting. The dishes with DRBC were incubated at 25°C for seven days, in absence of light. All dishes were analyzed, having selected those which presented CFU.g⁻¹ around 10 and 100. After the counting, the fungi colonies that were selected to identification were isolated and kept until the transplant to their correspondent medium, that was proper to each genus/specie, that is Spezieller Nalvistoffarmer Agar (SNA) for the genus *Fusarium* and Agar Estrato de Malte (MEA) for the genus *Aspergillus* and *Penicillium*.

The fungi colonies belonging to the genera *Aspergillus* and *Penicillium* were identified using identification keys described by Klich and Pitt (2002), based on sowing in four basic mediums: Czapek yeast extract agar (CYA); malt extract agar (MEA); Czapek yeast extract agar 20% sucrose (CY20S) and Agar 25% Glicerol Nitrate (G25N).

A conidial suspension was prepared from each strain in 0.5 mL of a medium constituted of 0.2% of Agar-agar and 0.05% of Tween

80TM, distributed in hemolysis tubes and previously sterilized at 121°C for 5 min (Pitt and Hocking, 2009). Then, a needle made of platinum was inserted into the conidial suspension and then transferred to three equidistant points in dishes containing CYA, MEA, CY20S and G25N. These dishes were incubated for seven days at 25°C. Each strain was identified accordingly to the methods described by Pitt (1988) and Klich and Pitt (2002).

The HMF data underwent the *Kolmogorov-Smirnov* K-S proof for normality, and then underwent analyzes of variance. For the variables related to the microbiological parameters, ANOVA test was performed with normalized data transformed to $\log_{10}^{(x+1)}$ and the F test was used to confront the existence of relevant differences between the average of the variables between the treatments. The Tukey's test at a significance level 5% was used to compare the averages, in accordance to the procedures established by the Statistical Analyses System (SAS, 1986). Frequencies of identification of the fungi genus and specie were calculated with the use of SPSS software, version 13.0.

RESULTS AND DISCUSSION

Results of physicochemical analyzes were expressed by means of average calculation and standard deviation and compared between the treatments (T1 and T2), and to the values suggested by the 11th Normative Instruction of the Agriculture and Supply Ministry of Brazil, according to the Table 1 (Brasil, 2000). Except from acidity in T2, the samples of T1 and T2 were within the limits established by the Brazilian legislation.

The variables of moisture, water activity and acidity showed differences between the treatments (T1 and T2) in the samples of honey of *A. mellifera* bees of the semi arid region of Piau . The average values for moisture were below 20% (Table 1) in accordance to the Brazilian legislation (Brasil, 2000), and were lesser than those found in the same region by Silva et al. (2004), who reported average values of 19.4% in the honeys associated to different studied crops. The results of moisture obtained in Northeast region varied between 17 and 20%, with average of 19.2% in honeys from the city of Crato, State of Cear  (de Araujo et al., 2006) and 18.7% in the other cities of that State (Sodr  et al., 2007), and in the State of Para ba (18.8%) (Rodrigues et al., 2008).

There were difference in moisture values between the

results of the two treatments of this study ($p < 0.05$) and the samples of T2 with the largest percentiles (Table 1). The Africanized bees cap the honey when moisture is between 17 and 18% (Evangelista-Rodrigues et al., 2005). This indicates maturity (Brasil, 2000). This parameter of quality also can influence directly the stability of the honey and the microbial changes by the contamination attributed to the bees, the nectar, the environment and the inadequate handling during the whole processing of honey. The quantity of microorganisms associated to moisture can favor the fermentation when the temperature is high and the storage is made in improper conditions. On the other hand, when analyzing this parameter, the samples of the honeys recently collected by the beekeeper offer probable stability from the microbiological point of view.

The result for the water activity (a_w) showed an average value of 0.68 and 0.76, for T1 and T2 ($p < 0.05$). Despite the fact that a_w is not an obligatory parameter for quality evaluation according to the Brazilian legislation (Brasil, 2000), this parameter, together with the levels of moisture, assigns a better protection against the growing of microorganisms. Values over 0.60 may favor the growing of xerophilic fungi and osmophilic yeast, in addition to halophilic bacteria (Jay, 2005; Franco and Landgraf, 2008). The hygroscopic character of the honey favors the absorption of water in environments where the relative humidity of the air (RH) is superior to that of the honey. Even though the RH of the semi-arid region of Piauí (Brasil, 2005) does not favor the increasing of a_w in the honey - since usually the honey handling is made in a hot and dry atmosphere, which is typical of the region - the average values of a_w found in the samples are superior. This may be related to improper storage conditions of the honey after its extraction.

Moreover, these values have been shown to be superior when compared to the average values of a_w of 0.58 to 0.60 found by Moura et al. (2014) that evaluated honeys in the semi arid region of Piauí; to the values of 0.49 to 0.66 in honeys from São Paulo (Denardi et al., 2005); to the Schalabitz et al. (2010) that described values between 0.54 to 0.62 in the region of Taquari Valley, in the State of Rio Grande do Sul; a_w of 0.55 Moroccan honeys (Malika et al., 2005), and of Kacaniová et al. (2007), that found values between 0.46 to 0.66 in honeys from Slovakia.

There are no Standards defined for pH in current Brazilian legislation (Brasil, 2000). Nevertheless, this parameter is important to help in acidity evaluation and, in some degree, to foresee honey quality. There was no difference in pH values between both treatments whose average values found were 3.72 and 3.52 for T1 and T2, respectively (Table 1). They have shown numerically similar results from those obtained in studies with honeys of the Northeast region of Brazil (Silva et al., 2004; Evangelista-Rodrigues et al., 2005; Rodrigues et al., 2008), of the South region of Brazil (Mendonça et al.,



Figure 1. Average light-amber color found in studied honeys.

2008; Welke et al., 2008) and over the world (Iurlina and Fritz, 2005; Malika et al., 2005; Tchoumboue et al., 2007). Such values may be attributed to different flowering composition, as well as nectaries, as Crane (1983) pointed out. Comparing with previous Brazilian legislation (Brasil, 1985) it can be noticed that the results of this study are in accordance with the recommended.

Regarding acidity, there were differences between the treatments ($p < 0.05$), with T2 showing higher averages (Table 1) with 55.5% of the samples being above the limit established by the current Brazilian legislation (Brasil, 2000). The Brazilian norm established a maximum value of 50.0 meq.kg^{-1} , and the higher values indicate a high possibility of unwanted fermentation occurrence in honey. The results found in T2 were superior to that found by Sodr e et al. (2007) that evaluated honeys from Piauí; Rodrigues et al. (2008) that observed values of acidity in honeys from the State of Para iba; Finola et al. (2007), observing honeys from Argentina.

The color of honeys studied was similar in both treatments (Table 1) with predominance of a light amber color (Figure 1). These values were in accordance with the 11th Normative Instruction (Brasil, 2000) which established that the color of the honey can vary from water-white to a dark amber color. So, the honeys from treatments 1 and 2 had a color that was attractive to the market, in a way that it characterizes the product as well valued by the foreign market (Brasil, 2009). It is of note the preference of the honey colorization by the Brazilian consumers, once the Brazilian honey is always dealt in a way that it can satisfy the European consumer preferences. This predominance of light amber color was also observed by Mendonça et al. (2008) and Moura et al. (2014).

Regarding hydroxymethylfurfural (HMF) there were no difference between the treatments T1 and T2 (Table 1) with the values being within limits established by the

Table 2. Microbiological parameters of honeys from *Apis mellifera* L. bees.

Treatments	<i>Salmonella</i> spp.	Coliforms 35 and 45°C (MPN.g ⁻¹)	Coagulase-positive <i>Staphylococcus</i> (CFU.g ⁻¹)	Mesophilic Heterotrophic Bacteria (CFU.g ⁻¹)	Yeast and filamentous fungi (CFU.g ⁻¹)
T1	Abs in 25 g	< 3.0	Abs in 0.1 g	1.22 ^b	2.03 ^b
T2	Abs in 25 g	< 3.0	Abs in 0.1 g	2.42 ^a	2.70 ^a

T1, Honey from beekeepers that use the EBUP with Best Practices for Beekeeping. T2, Honey from beekeepers that use the EBUP without Best Practices for Beekeeping. Abs = absence; MPN.g⁻¹ = most probable number per gram, expressed in logarithms of base 10; CFU.g⁻¹ = colony-forming unit per gram, expressed in logarithms of base 10; ^a = Averages followed by the same letter in the lines and in the columns do not differ from each other in the Tukey's test (p<0.05).

Brazilian legislation (Brasil, 2000). The HMF value can identify deterioration of the honey related to the period of storage, sugar addition, pH, moisture and temperature conditions to which it underwent, apart from adulteration (White Junior, 1989, 1992). Therefore, HMF values of T1 and T2 samples of honeys suggest that they were not subjected to high temperatures and its analyses were performed quickly. The results obtained were close to those found by Sodr e et al. (2007) in honeys from the State of Cear a; Bera and Almeida-Muradian (2007), in honeys from the State of S o Paulo; and inferiors to those found by Arruda et al. (2005), in honeys from the State of Cear a; de Ara ujo et al. (2006); and Santos and Ribeiro (2009).

The results regarding the observed microbiological parameters are shown in Table 2. *Salmonella* spp. was absent from all samples of honey, and similar to those of Matuella and Torres (2000); Iurlina and Fritz (2005); Boff et al. (2008); Schlabitz et al. (2010); and Moura et al. (2014). Current Brazilian legislation (Brasil, 2000) does not determine microbiological parameters to honey, nonetheless the analysis of *Salmonella* spp. was the only microbiological parameter established by the previous legislation (Brasil, 1978). Bacteria like *Salmonella* spp. are capable of surviving in honey; however, they do not grow because of low values of water activity and pH (Snowdon and Cliver, 1996).

According to Table 2, in all samples analyzed the counting of coliforms at 35°C and coliforms at 45°C in both treatments results were lower than 3.0 MPN.g⁻¹ which evidenced security about the presence of coliforms and enteric pathogens. These results can be justified by the physicochemical composition of the honey, that determines which microorganism will be capable of growing or not. Similar results were found by Iurlina and Fritz (2005); Boff et al. (2008); Barros and Batista (2008) and Moura et al. (2014). Silva et al. (2008) associated the absence of these microorganisms to proper hygiene conditions during the processing of honey, guaranteeing the hygienic-sanitary quality of this product.

The counting of coagulase-positive *Staphylococcus* showed absence in 0.1 g of the sample (Table 2). These results are similar to those found by Matuella and Torres

(2000) and Schlabitz et al. (2010). The results of T1 and T2 showed that honey was obtained adequately, and that this product has inherent antimicrobial properties that reduce microbial growth and survival, and the properties of the high osmotic pressure and low water activity greatly contribute to this feature.

Microbiological analyzes in food are of fundamental importance for the prevention of diseases transmitted by them, and for the honey would not be different, since it is a widely consumed food in the world. The absence of *Salmonella* spp., coliforms at 35°C and 45°C and coagulase-positive *Staphylococcus* indicate that beekeepers, even when using Extraction Units for Bee Products without the Best Practices for Beekeeping (T2), kept the honey free of enteric bacteria, what represents a higher security to the consumer.

Factors such as moisture, aw and pH are limiting for the growth and development of microorganisms (Franco and Landgraf, 2008). Thus, the absence of the studied microorganisms may have been favored, both in T1 and in T2 (Table 1), by the moisture range, the water activity level and the pH level that were found in the analyzed samples, which are in conformity with the unfavorable conditions for such growth, especially regarding enteric bacteria, which in most cases can only tolerate an aw level of 0.86 and a pH level of 4.0 as a minimum.

There were differences between the treatments 1 and 2 regarding the count of mesophilic heterotrophic bacteria (Table 2). In the samples T1 and T2, the values in CFU.g⁻¹ were 1.0 x 10⁴ and 5.0 x 10⁴, respectively, and according to Franco and Landgraf (2008) in most foods the values above which sensorial changes can be detected are superior to 10⁶ CFU.g⁻¹. However, the results of the counting of these bacteria were numerically lower than those found by Iurlina and Fritz (2005); Barros and Batista (2008); Schlabitz et al. (2014). This counting is required to indicate the sanitary quality of foods; even if there have been no deterioration changes to them.

The results for fungi and yeast counting showed difference (p<0.05) between T1 and T2, with higher values for T2 (Table 2). However, when compared to other studies, they showed to be lower to those of Sodr e et al. (2007); Kacaniov a et al. (2007); Silva et al. (2008);

Table 3. Genera of fungi isolated from samples of *Apis mellifera* L. bees.

Fungi genus*	T1		T2		Total	
	N	%	N	%	N	%
<i>Penicillium spp.</i>	10	23.8	6	14.3	16	38.1
<i>Aspergillus spp. and telemorfos</i>	9	21.4	4	9.5	13	31
<i>Cladosporium spp.</i>	4	9.5	6	14.3	10	23.8
<i>Fusarium spp.</i>	1	2.4	0	0	1	2.4
<i>Byssochlamys spp.</i>	1	2.4	0	0	1	2.4
<i>Curvularia spp.</i>	1	2.4	0	0	1	2.4
Total	26	61.9	16	38.1	42	100

*Frequencies calculated with SPSS software of version 13.0. EUBP, Extraction Units for Bee Products; T1, honey from beekeepers that use the EBUP with Best Practices for Beekeeping.; T2, Honey from beekeepers that use the EBUP without Best Practices for Beekeeping. N, Numbers; %, values in percentile.

Table 4. Identification of fungi species in honeys from *A. mellifera* L. bees.

Fungi species*	T1		T2	
	N	%	N	%
<i>Aspergillus flavus</i>	5	33.3	1	8.3
<i>Aspergillus niger and agregattes</i>	2	13.3	3	25.0
<i>Penicillium citreonigrum</i>	3	20.0	0	0
<i>Penicillium decubens</i>	1	6.7	0	0
<i>Penicillium waskmani</i>	2	13.3	0	0
<i>Penicillium restrictum</i>	1	6.7	2	16.7
<i>Penicillium implicatum</i>	0	0	5	41.7
<i>Penicillium islandicum</i>	1	6.7	0	0
<i>Penicillium felutanum</i>	0	0	1	8.3
Total	15	100.0	12	100.0

*Frequencies calculated with SPSS software of version 13.0. EUBP, Extraction Units for Bee Products; T1, honey from beekeepers that use the EBUP with Best Practices for Beekeeping.; T2, Honey from beekeepers that use the EBUP without Best Practices for Beekeeping. N, Numbers; %, values in percentile.

Boff et al. (2008) and Lieven et al. (2009). These microorganisms can resist low levels of water activity and pH and for this reason they are usually found in honey.

Even though no sensorial changes associated to fermentation occurred in T2, the average acidity of 59 meq.kg⁻¹ in the samples (Table 2) may correspond to a parameter indicating fermentation by yeasts. This found may be associated to contamination by primary sources or to the observance of the Best Practices for Beekeeping during the handling of the hives, which emphasizes the importance of continuous monitoring of the processing of honey in order to guarantee that a safe food is commercialized.

However, despite the fact that the counting of filamentous fungi and yeast presented significant difference between T1 and T2 (Table 2), the frequency of genera and species of isolated fungi (Tables 3 and 4) showed contradiction between both T1 and T2. The numbers of fungi genera and species were higher in T1 than in T2, even though T1 is considered to have used the Best Practices for Beekeeping during the handling of

the hives. This fact can be conjectured when confronted to the parameter of $a_w > 0.60$ (Table 1), which according to Denardi et al. (2005) would be a limiting factor for the development of filamentous fungi.

From the 54 samples of honey analyzed, filamentous fungi were found in 42 samples (78.0%). The fungi prevalent in the samples of honey (Table 1) were *Penicillium spp.* (38.1%), *Aspergillus spp.* and their teleomorphs (31.0%), *Cladosporium spp.* (23.8%) and *Fusarium spp.* (2.4%), and T1 showed a higher quantity of fungi genera (61.9%). Similar results were found by Kacaniová et al. (2007), when in 30 samples of honey the prevalent genera were *Aspergillus*, *Penicillium* and *Cladosporium*, with the found values higher than those found in T1 and T2 in the semi arid region of Piauí, Brazil. Tchoumboue et al. (2007) reported that in 49 samples of honey from West Cameroon, 18.4% were *Aspergillus*. The presence of fungi in foods, specially of the genera *Aspergillus*, *Penicillium* and *Fusarium* are undesirable because some species are capable of producing enzymes spoilage; as well as production mycotoxins, that

are toxic products of the secondary metabolism and nowadays they represent contamination risk for the environment, which implies serious harm to human health (Corrêa et al., 1997; Bando et al., 2007).

Among the fungi species identified in this study, those that were found in most of the cases were *Aspergillus flavus* (33.3%), followed by *Penicillium citreonigrum* (20.0%) in T1; and *Penicillium implicatum* (41.7%), followed by *Aspergillus niger* and its aggregates (25.0%) in T2 (Table 4). Among these, the incidence of *A. flavus* in the honeys of T1 (Table 4) must be highlighted and evaluated with caution, since this species is capable of producing aflatoxin (Klich and Pitt, 2002).

However, the presence of filamentous fungi which produce mycotoxins does not necessarily indicate their presence in the studied food, since, in order to produce mycotoxin, microorganisms require enabling environments such as those with inadequate values and/or high levels of moisture, water activity and pH (Franco and Landgraf, 2008).

Nevertheless, according to the physicochemical characteristics analyzed (Table 1), it is possible to notice that the honeys of T1 did not showed enabling conditions to the development and the multiplication of fungi, as well as to the production of mycotoxins.

Martins et al. (2003) identified three fungi genera (*Aspergillus*, *Penicillium* and *Mucor*) and two genera of yeast (*Sacharomyces* and *Candida*) in honeys from Portugal, including specially *A. flavus* (57.5%), followed by *A. niger* (51.3%); and *Penicillium* spp. and *Mucor* sp. were isolated in 38.8 and 31.3% of samples, respectively. Aflatoxins, however, were not present in the samples due to the fact that honeys do not offer an enabling medium to the development of secondary metabolites of the multiplication of those microorganisms.

Snowdon and Cliver (1996) affirm that bacterial spores, filamentous fungi and yeast can be acquired from primary sources related to the bees, or can be incorporated during the processing of the honey. The quality of honey can be affected by management during harvest. The quality of honey can be affected by handling during harvest. Thus, the beekeeper must perform appropriate procedures from the time of the harvesting of honey from beehives to its transport to the extraction unit, in order to interfere as little as possible in hygienic and sanitary quality.

Thus, the smaller the adoption of Best practices for beekeeping the higher is the contamination by microorganisms, especially by filamentous fungi and yeast, coupled with high levels of moisture and water activity which represent lower stability of the medium and high sanitary risk.

Conclusions

The quality of honey from *A. mellifera* L. bees produced in Piauí, Brazil was in conformity to moisture, water

activity, pH and HMF parameters, especially in samples from beekeepers that use the EUBP. The honey did not show contamination by *Salmonella* spp., coliforms and coagulase-positive *Staphylococcus*. Given the results of mycotoxin-producing fungi in the honeys, it is suggested that producers have greater concern for the quality control and use of BPB in all honey production steps.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Effect of processing steps and storage on microbiota and volatile amines content of salted semi preserved cuttlefish (*Sepia officinalis*)

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Microbial and volatile amines changes during processing steps and storage in optimal and abuse temperatures of semipreserved cuttlefish and their efficiency for quality was investigated. After osmotic treatment the initial microbial contamination and the total volatile basic nitrogen (TVBN) decreased sharply. The microbiota changed to Gram positive bacteria in which species of *Bacillus* and *Staphylococcus* were dominant. Mesophilic and psychrotrophic microorganisms increased markedly especially in abuse storage temperature and consequently chemical substances derived from its activity as total volatile basic nitrogen and ammonia. Among these parameters, mesophilic and psychrotrophic counts higher than 10^4 and 10^5 cfu/g, respectively, and TVBN contents higher than 100 mg per kilogram (mantle) or per liter (brine) would indicate a rupture of cold chain. Spanish legal microbial limit established is not appropriate for quality evaluation of semi preserved cuttlefish. A value of ammonia in muscle cuttlefish higher than 130 mg/kg would be useful spoilage indicator.

Key words: Cuttlefish, semi preserved microorganisms, total volatile basic nitrogen (TVBN), ammonia, Trimethylamine (TMA), Sepia.

INTRODUCTION

After years of gradual increase of the demand for cuttlefish with the depletion of oceanic fish stocks, the cuttlefish market has remained stagnant and international trade has declined into all main markets in 2013 (Japan, Italy and Spain, respectively by 18.5, 18 and 16.1%)

(FAO, 2014). Common cuttlefish usually is marketed fresh or especially frozen and it is a highly valued food item, especially in Japan, Korea, Italy and Spain (Reid and Roper, 2005). The main suppliers for these countries are Vietnam and Thailand for the Asian market and

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Europe and North African countries (France, Spain, Tunisia, Morocco, Senegal and Mauritania) for the European market (FAO, 2014). Despite these trends, cuttlefish are valuable and expensive fishery products and are important for economies of these countries (Caglak et al. 2014).

Cuttlefish is known to have a short life cycle and it is characterized by a high growth rate (Caglak et al., 2014; Sykes et al., 2009; Vaz-Pires et al., 2008). This high endogenous enzymatic activity allows a rapid alteration after death and hence favoring proliferation of spoilage microbiota which participate in the rapid alteration (Hurtado et al., 2001; Pacquit et al., 2006). The spoilage process in cephalopods is different from fish (Howaida, 2010). Cephalopods muscle tends to remain microbiologically less invaded until later than fish species as reported by Vaz-Pires et al. (2008). Total viable count and volatile compounds such as trimethylamine (TMA), dimethylamine (DMA) and ammonia are products of microbial degradation (Olafsdottir et al., 1997) and are used for microbial and chemical evaluation.

The FAO (2013) reported the lack of knowledge regarding improvement of fish handling and post-harvest practices. Most of the studies on methods to assess quality changes have been directed to several species of squid (Lapa-Guimaraes et al., 2002), and other cephalopods (Ruiz-Capillas et al., 2002), but no studies have examined quality changes and storage stability of semipreserved salted cuttlefish during process and storage despite its abundance and commercial importance. Lapa-Guimaraes et al. (2005) reported the importance to study the efficiency of tests in different storage conditions for cephalopods and to verify their correlation with microbial contamination, to guarantee consumer safety. Howaida (2010) pointed out the need to focus future research on cephalopods shelf-life under different storage conditions.

The main goal of this research was to generate information and to study changes in microbial, and volatile amines associated to microbial spoilage during processing steps and storage in optimal (4°C) and abuse temperature (simulating market conditions) of semipreserved salted cuttlefish that could be used by processors for better handling and maintenance of quality.

MATERIALS AND METHODS

Sample selection and processing

The species of cephalopod used in this study was cuttlefish (*Sepia officinalis*) with an average dorsal mantle length of 20 cm that was caught by artisanal boats on the coast of Senegal (located on the Atlantic Ocean). The product was taken on the same day to a factory where it was gutted, cleaned, skinned, rinsed, frozen at -30°C and stored in the freezer at -20°C. This product was weekly sent to Spain to a local factory and processed to semipreserved cuttlefish as described below. Frozen raw material was completely

thawed in running tap water at about 20°C. After that, cuttlefish was brined by immersion in moving air in 5.6% salt solution at about 20°C during 15 min. Thereafter, in order to tenderize the product, cuttlefish was immersed in 4.8% salt solution with additives (sodium sorbate) at 0-3°C for 48 h, representing maturing process or osmotic treatment. After this treatment cuttlefish was packaged in polyethylene bags of 3 kg of capacity, with cuttlefish, brine and ice in a proportion of 1:1:1. Frozen (F), thawed (T) and brined (B) cuttlefish were stored in clean isotherm container and the plastic bags containing semipreserved cuttlefish were stored in 15 separate isothermal polystyrene boxes. All samples were transported to the laboratory within a maximum period of 45 min. One box was separated for the first sampling (day 0) and the others were randomly divided and submitted to two different storage temperatures. Eight boxes were stored at refrigeration temperature (4°C, optimal temperature) and analyses were performed after days 2, 6, 10, 12, 14, 16, 18 and 20. The remaining six boxes, simulating the market conditions, were stored at 20°C during 6 h and at 4°C during the rest of the day (abuse temperature). Samples were periodically taken on days 2, 4, 6, 8, 10 and 12 for physico-chemical, biochemical and microbiological analysis. The whole experiment was repeated five times.

Microbiological analysis

Bacterial counts

Each sample (10 g) was taken aseptically and homogenized mechanically with 90 ml of buffered peptone water and appropriate dilutions were prepared. Total psychrotrophic and mesophilic aerobic microorganisms (Valle et al., 1998) were enumerated on plate count agar (PCA) (Oxoid, England) containing 0.5% NaCl (Panreac, MontcadaiReixach, Spain) at 20°C for 72 h and at 30° for 48 h, respectively, and enterobacteria in violet red bile glucose (VRBG) (Oxoid) at 37°C for 24 h (Vanderzant and Splittstoesser, 1992). Counts were performed in duplicate and expressed as log of cfu/g.

Isolation, purification and identification of microorganisms

All plates of PCA with countable colonies were examined for typical colony types and morphological characteristics. Five colonies were randomly isolated and purified by successive sub-culturing on trypticase soy agar (Oxoid) until pure colonies were obtained. Strains were identified on the basis of Gram reaction, catalase and citochromooxidase production. Further identification to the species level was accomplished by a variety of biochemical tests using the API-20 NE (BioMerieux, Marcy l'Etoile, France) for Gram negative isolates and API-Staph and API-50-CHB (BioMerieux) for Gram positive isolates.

Physico-chemical analysis

Prior to analysis, mantle part of cuttlefish was minced. All chemical tests were done in duplicate.

pH and volatile amines

Prior to analysis, mantle part of cuttlefish was minced. The pH measurements was done with a digital pH-meter (Crison 2001, Crison Instruments, Barcelona) by placing the calibrated electrode into the minced and homogenized cuttlefish mantle and into the brine.

TVBN was estimated by perchloric acid extraction and steam

distillation (Anonymous, 2005). The volatile nitrogenous bases were extracted from a sample (minced cuttlefish and brine) using a solution of 6% perchloric acid. After filtration and alkalisation the extract underwent steam distillation performed in a TecatorKjeltec model 2200 (Foss, Sweden). The TMA content of the semipreserved cuttlefish was determined by the modified Dyer picrate method (AOAC, 2003). DMA was determined by the copper-dithiocarbamate method (Dyer, 1945).

Ammonia and monomethylamine (MMA) were determined by the high pressure liquid chromatography (HPLC) method of Valle et al. (1996). Cuttlefish muscle (5 g) was homogenized with 10 ml of 0.2N perchloric acid and 100 µl of 1.3-diaminopropane solution (80 mg/100 ml; interne standard) with an Ultra-Turrax mixer (Heidolph, Germany) at maximum speed and centrifuged at 2500 g for 10 min at 4°C. The supernatant was filtered through Whatman n° 1 filter paper, the pellet was mixed with 10 ml of 0.2 N perchloric acid and the same operation was repeated, the resultants filtrate were frozen and preserved at -20°C (for a maximum of 2 months) until use. Volatile and biogenic amines were detected as dansyl derivatives extracts that were filtered through a 0.22 µm nylon filter into the HPLC sampling vial and were then used directly for analysis. Chromatographic separations were carried out with a HPLC-LC Module I Plus (Waters Cromatografía, España), 20 µl was injected with a flow of 0.8 ml/min using C18 5-µm-particle-size (4.6 mm x 15 cm) reversed-phase analytical column controlled by a thermostat set at 25°C. 1-3, diaminopropane-2HCl was used as the internal standard to evaluate losses. Quantification of the volatile amines was carried out with the data module in the internal and external standardization modes.

Statistical analysis

Results were evaluated by one-way ANOVA, where the main effects were temperature (optimal and abuse) and storage time. Student-Newman-Keuls post-hoc tests were used as paired comparisons between samples means (Bower, 1998). Simple linear correlation (Pearson correlation) analysis was done between different parameters (Bower, 2000). Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS Inc. Released 2009. PASW Statistics for Windows, Version 18.0. Chicago: SPSS Inc). Significance of differences was defined at $p < 0.05$.

RESULTS AND DISCUSSION

Microbial characterization

Mesophilic, psychrotrophic and enterobacteria counts decreased significantly ($p < 0.05$) after the osmotic treatment probably because of combined effect of salt (4.8% NaCl), additives and temperature (0-3°C) during this stage (48 h). For semipreserved cuttlefish stored in optimal temperature, mesophilic and psychrotrophic counts reached at the end of storage were approximately 3.8 and 4.8 log cfu/g, respectively. However negative sensory characteristics were observed after day 14th-16th of storage, suggesting the enzymatic action is more rapid in cuttlefish than bacterial spoilage action. On the other hand, in abuse temperature psychrotrophic, mesophilic and enterobacteria counts increased sharply ($p < 0.05$) from 6th day of storage and reached respectively 7.3; 6.4 and 1.8 log cfu/g at the end of storage (Figure 1). Day 6th was considered to be the rejection point. The legal limits

for mesophilic bacteria and enterobacteria for frozen, iced and slightly salted fish products iced or frozen fish is 10^6 and 10^3 cfu/g, respectively (Anonymous, 1991). The microbiological quality of frozen cuttlefish was very good in accordance with the legal limits (Figure 1). These results are very similar to those obtained by Lapa-Guimaraes et al. (2002) who observed that iced stored cephalopods have a spoilage pattern dominated by autolysis and a very late microbial growth. These results suggest that mesophilic and psychrotrophic counts higher 10^4 cfu/g and 10^5 cfu/g, respectively, would indicate a rupture of cold chain. Moreover, we can conclude that the spanish legal microbial limit established is not appropriate for quality evaluation of semipreserved salted cuttlefish.

During the iced storage of whole cephalopods, a heterogeneous microbiota containing *Shewanellaputrefaciens*, *Pseudomonas* spp. and *Photobacterium phosphoreum* are the dominant spoilage bacteria (Lapa-Guimaraes et al., 2002; Howaida, 2010). However, in our study microbiota was dominated by Gram positive bacteria (94.5%). The Gram positive were especially halotolerant-halophilic bacteria *Bacillus* (39.8%), *Staphylococcus* (32%), *Micrococcus* (11.7%), and *Aerococcusviridans* (9.4%). The high salt concentration leaves only salt tolerant microorganisms to survive; salt concentration up to 7% results in the inhibition of lactic acid bacteria (Horner, 1997). In our study 5% of salt was used for brine solution, however lactic acid bacteria were not identified because probably of the presence of additives. Gram negative bacteria were *Pseudomonas* spp (3%), *Pantoea* spp (1.6 %) and *Acinetobacter* spp (<1%) (Table 1). In fish products preserved by the addition of low levels of NaCl respiratory Gram-negative bacteria are typically inhibited (Gram and Dalgaard, 2002). Centeño-Briceño and Rodríguez-Bejarano (2007) observed in frozen squid *Achromobacter*, *Shewanella putrefaciens*, *Acinetobacter* and *Aerococcus* as dominant psychrotrophic bacteria, while *Micrococcus* and *Acinetobacter* were dominant between mesophilic bacteria. Moreover, Gram-positive *Bacillus* and *Micrococcus* dominate on fish from tropical waters (Shewan, 1977) in accordance with our raw material which is from tropical water Atlantic Ocean (Senegal).

As part of the qualitative characterization of the isolates from spoiled semi preserved cuttlefish, dominant microorganisms were *Bacillus megaterium* for 27.3% of the *Bacillus* population. *Bacillus subtilis/amyloliquefaciens* and *Bacillus cereus* accounted for 5.5 and 4.7% respectively (Table 1). The presence of *Bacillus* spores in other fish products at the level of 10^3 - 10^4 cfu/g was reported by Dube et al. (2004). *Staphylococcus* species consisted mainly of *Staphylococcus hominis* (14.1%) and *Staphylococcus warneri* (7%) of the *Staphylococcus* population (Table 1). *Bacillus megaterium* and *Staphylococcus hominis* which seem to be favoured by treatment are recognized as causing spoilage of foods

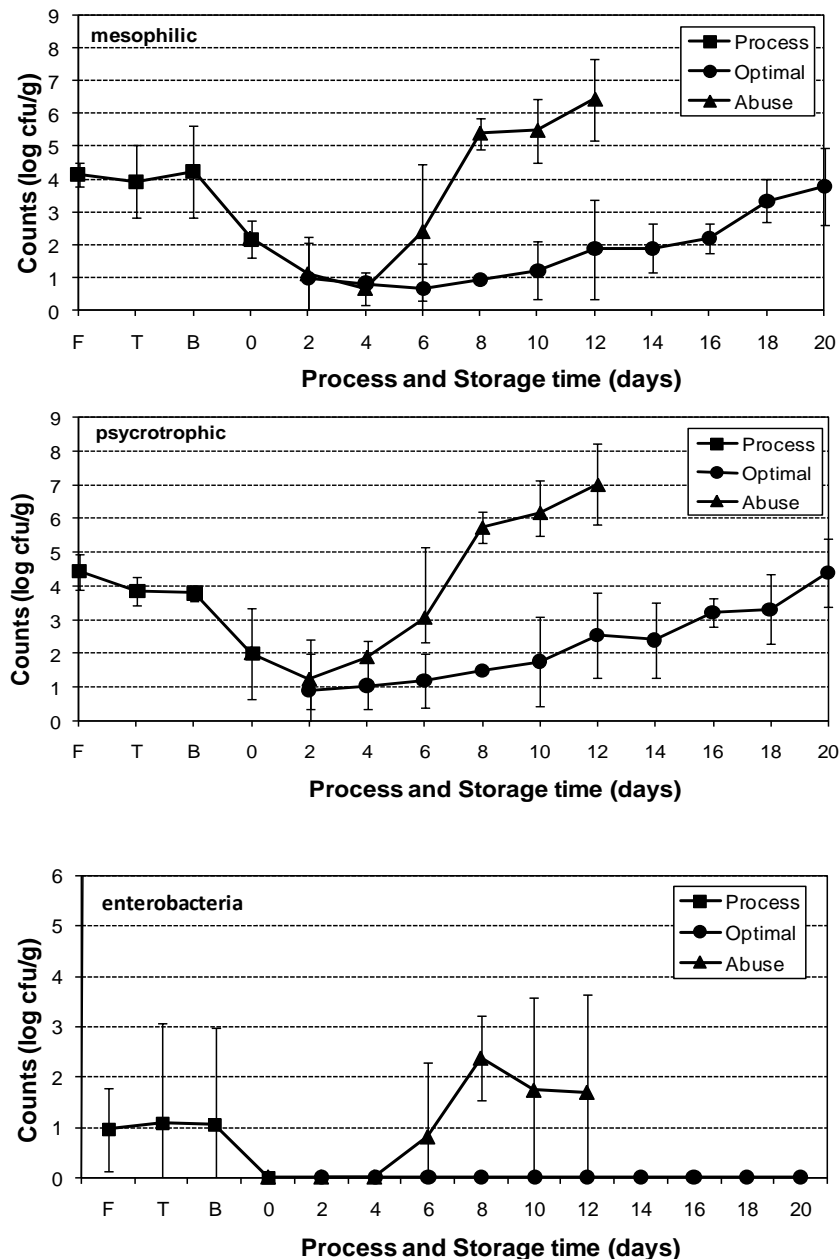


Figure 1. Microbiological changes during process (F: frozen; T: thawed with water; B: brined) and storage time in optimal and abuse temperatures (mean \pm standard deviation).

(Kraft, 1992; Ray, 2001). The spoilage activity of bacteria varies depending on the hydrolytic enzymes that they produce. In fact, *B. subtilis* and *B. megaterium* have been described as microorganisms with a high proteolytic activity (Anihouvi et al., 2007). *Micrococcus* and *Bacillus* may be present in large quantities without producing significant organoleptic changes (Chen, 1995), however, their contribution could be important for spoilage and specially in abuse storage temperature, because they represented 73% of the microbiota identified.

pH and volatile amines

The pH values of recently captured cephalopods fluctuate between 5.9 and 7.0 (Yamanaka et al., 1995; Ruiz-Capillas et al., 2002; Marquez-Rios et al., 2007; Howaida, 2010). In our study, the initial pH value of frozen raw material was around 6.51, after osmotic treatment pH value increased to 6.63 and varied depending on the storage temperature (Table 2). In post-mortem cold stored marine invertebrate muscle the pH change is

Table 1. Percentage of mesophilic and psychrotrophic microorganisms isolated from semi preserved cuttlefish.

Parameter	Species	Isolates number	%
Gram positive (94.53%)	<i>Bacillus megaterium</i>	35	27.34
	<i>Staphylococcus hominis</i>	18	14.06
	<i>Aerococcus viridans</i>	12	9.38
	<i>Micrococcus</i> spp.	12	9.38
	<i>Staphylococcus warneri</i>	9	7.03
	<i>Bacillus subtilis/amyloliquefaciens</i>	7	5.47
	<i>Bacillus cereus</i>	6	4.69
	<i>Staphylococcus equorum</i>	4	3.13
	<i>Staphylococcus lugdunensis</i>	3	2.34
	<i>Bacillus non-reactive</i>	2	1.56
	<i>Micrococcus luteus</i>	2	1.56
	<i>Staphylococcus epidermidis</i>	2	1.56
	<i>Bacillus firmus</i>	1	0.78
	<i>Brevibacillus non reactive</i>	1	0.78
	<i>Bacillus lentus</i>	1	0.78
	<i>Micrococcus lysae</i>	1	0.78
	<i>Staphylococcus cohnii</i>	1	0.78
	<i>Staphylococcus hyicus</i>	1	0.78
	<i>Staphylococcus simulans</i>	1	0.78
	<i>Staphylococcus capitis</i>	1	0.78
<i>Staphylococcus caprae</i>	1	0.78	
Gram negative (5.47%)	<i>Pseudomonas</i> spp.	4	3.13
	<i>Pantoea</i> spp.	2	1.56
	<i>Acinetobacter</i> spp.	1	0.78

Table 2. pH value changes during storage in abuse temperature (mean \pm standard deviation).

Parameter	Mantle		Brine	
	Optimal	Abuse	Optimal	Abuse
Frozen	6.51 \pm 0.12 ^a	-	-	-
Thawed	6.50 \pm 0.08 ^a	-	-	-
Brined	6.54 \pm 0.09 ^a	-	-	-
0	6.63 \pm 0.02 ^b	-	7.17 \pm 0.18 ^a	7.17 \pm 0.18 ^a
2	6.78 \pm 0.15 ^c	6.85 \pm 0.04 ^c	7.00 \pm 0.07 ^b	6.89 \pm 0.01 ^a
4	-	6.87 \pm 0.12 ^c	-	6.94 \pm 0.10 ^a
6	6.91 \pm 0.10 ^{cd}	6.96 \pm 0.11 ^d	6.92 \pm 0.01 ^b	6.82 \pm 0.12 ^a
8	-	6.80 \pm 0.28 ^c	-	6.43 \pm 0.38 ^b
10	7.06 \pm 0.06 ^d	6.68 \pm 0.39 ^b	6.97 \pm 0.08 ^b	6.31 \pm 0.37 ^b
12	6.95 \pm 0.10 ^d	6.77 \pm 0.10 ^b	6.90 \pm 0.04 ^b	6.49 \pm 0.05 ^b
14	6.98 \pm 0.06 ^d	-	6.92 \pm 0.04 ^b	-
16	7.01 \pm 0.10 ^d	-	6.96 \pm 0.09 ^b	-
18	6.94 \pm 0.07 ^d	-	6.98 \pm 0.01 ^b	-
20	6.98 \pm 0.13 ^d	-	6.99 \pm 0.10 ^b	-

^{a-d}Values in same column followed by different letters were significantly different (p<0.05).

related to the increase in both lactic acid and octopine contents (Kawashima and Yamanaka, 1995). In cephalopods and other mollusks, octopine is the end-product from the anaerobic metabolism and is less acidic (Livingstone, 1983; Shin et al. 1998). The synthesis of octopine is catalyzed by the enzyme octopine dehydrogenase with an optimal pH for activity around 6.5 (Gade and Grieshaber, 1986), thus the post-mortem pH detected in cephalopods muscle promotes its formation (Marquez-Rios et al. 2007). This result was in accordance with others authors such as Ohashi et al. (1991) and Yamanaka et al. (1987) in fresh squid stored in refrigerate temperature. Similar results were found by Chiou et al. (2002) in abalone stored at 15 and 25°C. They observed a significant decrease of pH value although the TVBN content increased. When abalone was stored at 5°C the pH value remained constant. In contrast, Yamanaka et al. (1987) observed a marked increase of pH value and TVBN content in squid stored at 15°C and Calgak et al. (2014) observed an increase of pH during the storage period of cuttlefish at 3°C from 6.32 to 7.65. Although some authors suggested pH value as a useful index for the stage of initial decomposition, this parameter was not useful for semipreserved cuttlefish.

Volatile amine determination showed a high variability; possibly due to different seasonal periods in which was carried out the sampling that could substantially affect the chemical composition initial (Figure 2 and Table 3). There is currently no legal TVBN limit for cephalopods and the limit for fish 250-350 mg/kg is used (Huss, 1995; Anonymous, 2005; Gulsun et al. 2009; Amegovu et al. 2012). Results of the current study show that the quality of frozen cuttlefish (220 mg/100 g) was very good and it would belong to the grade A category. However, this content was higher than level observed in other studies carried out in cephalopods (Lapa-Guimaraes et al. 2005; Ruiz-Capillas et al. 2002; Yamanaka et al., 1995). In the Japanese market, a TVBN value above 150 mg/kg is considered as unacceptable (Ruiz-Capillas et al., 2002).

The TVBN quantified in marine products has been used as indicator of bacterial spoilage and as quality indicators (Vaz-Pires et al., 2008; Zhong-Yi et al., 2010; Amegovu et al., 2012; Farida et al., 2014). The increase in TVBN content may be due essentially to the bacterial action, particularly by the growth of psychotropic bacteria. However, endogenous production of ammonia from the enzymatic degradation of proteins, amino acids, adenylated nucleotides and nucleosides (Huss, 1995), could generate an initial postmortem content of TVBN in the range from 80 to 120 mg/kg muscle (Pivarnik et al., 1998). TVBN levels in iced cephalopods are much higher than those in fish, probably because of the high composition of volatile compounds (Ruiz-Capillas et al. 2002; Yamanaka et al., 1995). Caglak et al. (2014) found a value of TVBN which exceeded acceptability limits on day 5 for cuttlefish stored with ice at 2±1°C. Then, values of limits proposed for fresh cuttlefish are not applicable

for semi preserved salted cuttlefish. According to our results, 100 mg per liter or per kilogram of TVBN content in brine or mantle, respectively, would indicate a rupture of cold chain. Analysis in the brine would allow one to obtain the samples by non-destructive methods.

Initially ammonia content in relation to TVBN was about 53% coinciding with the values obtained by other authors in squid (Lapa-Guimaraes et al. 2005). Statistical differences ($p < 0.05$) were observed in ammonia content depending on storage temperature. Some authors observed an ammonia production at low bacterial cell densities, indicating that autolysis was causing the production during first period of storage (LeBlanc and Gill, 1984; Paarup et al. 2002). Ammonia production increased in a later stage because of bacterial activity. Similarly, others authors observed a final exponential increase of ammonia in whole iced squid (Licciardello et al. 1985). Its formation seems to derive from the deamination of the amino acids and of the urease bacterial activity (Paarup et al., 2002). Ammonia has been proposed as an objective quality indicator for cephalopods which degrade autolytically rather than primarily through bacterial spoilage (LeBlanc and Gill, 1984). According to Licciardello et al. (1985), the ammonia could be an indicator of cephalopods spoilage. In semipreserved cuttlefish a value higher than 130 mg/kg would be a useful spoilage indicator.

Higher initial trimethylamine-oxide (TMAO) contents (80-470 mg/100 g) have been found in common cuttlefish (*Sepia officinalis*) and several squid species of the *Loligo*, *Illex* and *Todarodes* genera (Boumpalos and Lougovois, 2005; Lapa-Guimaraes et al., 2005), so the potential for TMA formation in cuttlefish is high; some authors suggest it as an indicator for spoilage (Caglak et al., 2014). Vaz-Pires et al. (2008) reported a TMA value of 0.3 and 10 mg/100 g for respectively fresh and spoiled cuttlefish after 10 days in ice storage. However, a low value of TMA content was observed in raw material and it remained constant during osmotic treatment and storage in optimal and abuse temperatures. Similar results were obtained by others authors in iced squid muscle (*Loligo plei*), iced octopus (*Eledone moschata*) and chilled pressurized octopus (*Octopus vulgaris*) (Hurtado et al. 2001; Lapa-Guimaraes et al., 2005). Extensive losses of TMAO, not related to bacterial reduction, have been reported for squid, common cuttlefish and octopus stored in melting ice (Boumpalos and Lougovois, 2005; Lapa-Guimaraes et al., 2005; Livingstone, 1983; Lougovois et al., 2008). For this reason TMA is not a particularly good indicator of edibility of cephalopods. Moreover, the TMAO reducing capacity has been found in *Aeromonas* spp., psychrotolerant *Enterobacteriaceae*, *P. phosphoreum*, *S. putrefaciens* like organisms and *Vibrio* spp (Gram and Dalgaard, 2002). *Bacillus* and *Staphylococcus* which apparently dominated the flora of spoiling semipreserved cuttlefish do not reduce TMAO, so that spoilage can occur with little or no TMA production.

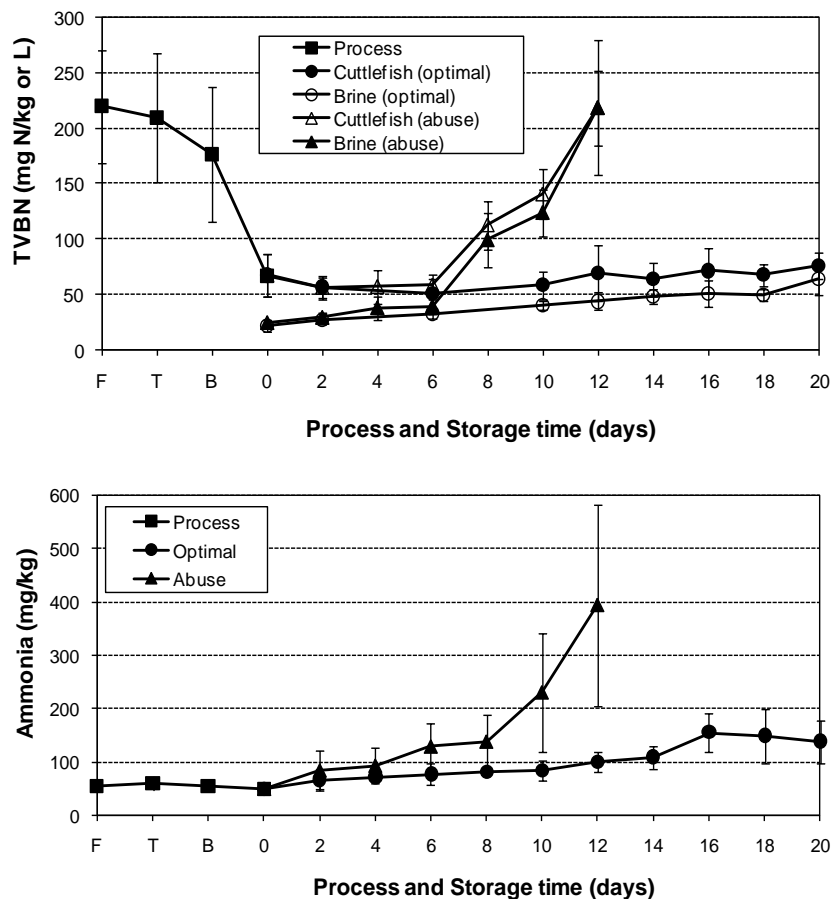


Figure 2. Total volatile basic nitrogen (TVBN) and ammonia changes during process (F: frozen; T: thawed with water; B: brined) and storage time in optimal and abuse temperatures (mean \pm standard deviation).

Table 3. Trimethylamine (TMA), dimethylamine (DMA), monomethylamine (MMA) and formaldehyde (FA) changes (mg/kg) during process and storage in optimal and abuse temperatures (mean \pm standard deviation).

Parameter	TMA		DMA		MMA	
	Mantle		Mantle		Mantle	
	Optimal	Abuse	Optimal	Abuse	Optimal	Abuse
Frozen	15.93 \pm 3.44	-	10.66 \pm 1.47 ^a	-	8.80 \pm 3.83 ^{ab}	-
Thawed	14.11 \pm 3.12	-	9.22 \pm 1.16 ^b	-	8.41 \pm 2.04 ^{ab}	-
Brined	13.74 \pm 2.87	-	8.57 \pm 0.84 ^b	-	6.96 \pm 2.75 ^{ab}	-
0	13.23 \pm 3.37	-	7.31 \pm 0.86 ^c	-	7.94 \pm 3.39 ^a	-
2	12.99 \pm 2.96	12.89 \pm 3.32	6.43 \pm 0.33 ^c	6.63 \pm 0.59 ^{cd}	10.06 \pm 1.67 ^a	11.20 \pm 3.98 ^{ab}
4	-	13.10 \pm 3.14	-	6.43 \pm 0.39 ^d	-	11.35 \pm 3.55 ^{ab}
6	12.88 \pm 3.55	13.03 \pm 3.02	5.84 \pm 0.11 ^c	5.88 \pm 0.27 ^d	15.31 \pm 5.64 ^a	16.39 \pm 3.45 ^{ab}
8	-	14.61 \pm 3.65	-	5.96 \pm 0.21 ^d	-	18.83 \pm 10.08 ^{ab}
10	13.07 \pm 3.33	14.07 \pm 3.30	6.18 \pm 0.35 ^{cd}	5.87 \pm 0.35 ^d	14.71 \pm 9.84 ^a	19.84 \pm 11.27 ^b
12	13.03 \pm 3.16	15.92 \pm 3.95	5.87 \pm 0.53 ^{cd}	6.08 \pm 0.18 ^d	15.65 \pm 5.55 ^a	18.35 \pm 2.82 ^b
14	12.96 \pm 3.49	-	5.41 \pm 0.32 ^{cd}	-	15.22 \pm 3.84 ^a	-
16	12.99 \pm 3.26	-	5.87 \pm 0.73 ^{cd}	-	14.27 \pm 2.19 ^a	-
18	13.03 \pm 3.53	-	5.13 \pm 0.27 ^d	-	15.29 \pm 5.68 ^a	-
20	13.77 \pm 2.62	-	5.18 \pm 0.38 ^d	-	14.32 \pm 3.47 ^a	-

^{a-d}Values in same column followed by different letters were significantly different ($p < 0.05$).

Certain types of fish contain an enzyme, TMAO dimethylase (TMAO-ase), which converts TMAO into equimolar quantities of DMA and formaldehyde (FA). DMA and FA production was analysed, since it was reported a TMAO-ase enzymatic activation under conditions of low oxygen availability in iced fish (Lundstrom et al. 1982). The initial DMA content of raw material was around 10 mg/kg. This value was ten times lower than the amount reported by (Kolodziejska et al. 1994) in frozen mantle of squid. This difference depends on species characteristics or treatment after catching. This initial increase is probably due to DMA bacterial degradation. *Micrococcus* spp, isolated in our study was described as having the ability to use DMA as a carbon source (Tate and Alexander, 1976). Aerobically, dimethylamine is oxidized to methylamine by dimethylamine mono-oxygenase. The methylamine produced from dimethylamine is further oxidized to yield ammonium by a methylamine mono-oxygenase (Kim et al., 2001).

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Bacteriophage based self-assembled monolayer (SAM) on gold surface used for detection of *Escherichia coli* by electrochemical analysis

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Bacteriophages (or phages) are parasites that infect specific bacteria. This host-selectivity can be useful to identify bacterial contaminants in food, water, environment etc. In the present study, phage was isolated from stagnant water and cultivated by overlay method against host bacteria that is *E. coli*. Phage titer was calculated to be 10^7 pfu/ml using 10-fold dilutions. Plaque reaction activity was observed within 4 to 6 h against host bacteria by spot test. Morphological identification of phage by transmission electron microscopy (TEM) using uranyl acetate staining revealed about 78 nanometers (nm) in wide phage capsid and tail length (527 nm). The isolated phage was classified into order *Caudovirales* since it possessed a long non-contractile tail and icosahedral capsid head, thus is a member of the family *Siphoviridae*. Also, the phage identified followed a lytic life cycle since plaque reaction activity was observed within 4-6 h against host bacteria. Gold immuno-functionalization using self-assembled monolayers (SAM) has been widely used for the detection of small targets, but there are limited reports available describing the detection systems for bacteria by using phages. Thus, in order to develop a suitable detection system for identification of specific bacteria, it is suitable to exploit the close association and selectivity between bacteria and bacteriophage. Bacteriophages were immobilized onto gold surface by SAM using a stable acyl amino ester intermediate generated by 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) to condense the bacteriophage. Fourier transformation infrared (FTIR) microscopy presence of different functional groups present in each layer formation. Bacteriophage immobilization over the gold surface was verified through by scanning electron microscope (SEM). Electrochemical analysis was performed for a rapid and specific detection of *E. coli* cell. The present bio-sensing system comprises of quick and specific detection of host bacteria and possesses a very low detection limit (10^4 cfu/ml). We propose phages utilization as a bio-component in biosensor development for bacteria capture.

Key words: Bacteriophage, biosensors, electrochemical, microscopy, immune-functionalisation and Self-assembled molecular monolayer.

INTRODUCTION

Bacteriophages are highly specific viruses that infect bacteria and are essentially harmless to humans. High host-specificity and selectivity promotes distribution of

virions in locations populated by bacteria, such as natural water bodies, soil and intestinal tracts of warm-blooded animals.

Escherichia coli is a natural resident in the intestinal tracts of animals including humans. *E. coli* can cause diarrhea, food poisoning, inflammations etc. also major cause of foodborne illness (Geng et al., 2008).

Bacterial infection has been one of the major causes of outbreak of diseases and hence a major threat for human health and food safety for decades (Velusamy et al., 2009). Conventional methods for detection of these pathogens include colony counting, biochemical methods, immunological assays, PCR methods involving DNA analysis etc. These conventional methods may take from several hours to even a few days to yield an answer. Thus, there is an immediate need for developing sensitive, selective, reliable and quick bacterial sensing platforms which ensure pathogen detection at low concentration, and are affordable to use (Chemburu et al., 2005). In this regard, researchers are now-a-days focusing towards development of rapid detection methods (Petty et al., 2006; Alocilja and Radke, 2003; Naidoo et al., 2012).

Bacterial biosensing platform is based upon the use of detecting the presence of bacteriophage cross-linked to gold surface itself, instead of selective antibody identification. During infection, the bacteriophage binds to specific receptors on the host bacterial surface. Following injection of the phage genome into the bacteria, high copy-number replication of phage takes place in the host bacterial cell, and subsequently, several new phages are released. Phage production is possible in an easier, faster, and cheaper, which makes them promising tools in biosensing (Johnson and Zeikus, 1991; Balasubramanian et al., 2007; Zeikus and Johnson, 1991; Yang and Bashir, 2008). They can also be immobilized onto transducing devices which makes detection more rapid biosensor platforms being developed for electrochemical analysis by impedometric-based detection (Zourob, 2010; Rohrbach et al., 2012).

Electrochemical impedance spectroscopy (EIS) is of great attention since it is proficient and frequent in detecting small change occurring at the solution-electrode interface and also corresponds to the charge transfer resistance of the electrode/electrolyte interface. Furthermore, EIS is simple, cost effective and processing is less reagent-dependent (Mejri et al., 2011). Recently, use of EIS has been broadly applied in the field of microbiology as a means to sense and measure pathogenic bacteria (Mejri et al., 2011; Mejri et al., 2010; Rohrbach et al., 2012; Park et al., 2013).

Modification or functionalization means the transformation of a biologically-inert material into a bioactive or bio-functional material. When gold surface are considered, preferred strategies via self-assembled monolayer (SAM) and covalent binding provide options

for strong and stable biomolecules immobilization in the very near vicinity of the sensor surface (Yang and Bashir, 2008; Shabani et al., 2008; Mejri et al., 2010, Yang and Li, 2005; Yang et al., 2004; Ruan et al., 2002; Radke and Alocilja, 2005). As gold surface is relatively homogeneous and provides suitable reactive groups for the directed conjugation of capture biocomponents it is used as a substrate (Baldrich et al., 2008). Various researchers have used this strategy for bacterial detection and there have been many successful reports in which a SAM-based immuno-sensor enables the detection of bacteria (Patel et al., 1997; Baldrich et al., 2008; Geng et al., 2008; Schofield et al., 2012).

In present investigation, experiments were carried out to isolate and characterize the *E. coli* specific bacteriophage from waste-water sample. Isolated bacteriophage was immobilized on modified gold (Au) electrode by SAM method. Mercaptoundecanoic acid was used for self-assemble on Au surface to form an oriented monolayer. The immobilization of bacteriophage on the SAM was carried out through a stable acyl amino ester intermediate generated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) cross-linking reaction. The co-addition of EDC and NHS can provide the formation of an appropriate intermediate to condense bacteriophage on the SAM. Gold surface modification identify was by screening electron microscopy. This strategy allowed *E. coli* detection with a limit of detection of 10^4 CFU/ml by electrochemical impedance analysis.

MATERIALS AND METHODS

Bacteria

E. coli MTCC-1585 were grown in LB medium (HI-Media) for 16-20 h at 37°C. Cells were titered using culture plate colony count and found approximately to be 2×10^4 CFU/ml and served as stock culture. The stock culture (1 ml) was subjected to serial dilutions in sterile phosphate buffered saline (PBS, 120 mM NaCl, 50 mM NaH_2PO_4 , pH 7.4) (Sambrook et al., 1989).

Isolation of phage, enrichment and overlay method

Waste water sample were centrifuged out at 3000 to 5000 rpm for 15 min at room temperature to remove any large debris and insoluble waste. The supernatant containing phages was filtered through sterile filters (pore size 0.45 μm , Millipore) and was added to log phase *E. coli* culture broth for 5 to 24 h in a shaking incubator (200 rpm at 37°C) for phage growth (Sambrook et al., 1989). The broth was centrifuged at 5000 rpm for 15 min at room temperature and the supernatant was re-filtered to obtain the final enriched phage suspension (Sambrook et al., 1989; Jordan et al., 2011). The presence of phages in the enriched filtrate was validated by overlay method with some modifications (Adams, 1959). Briefly, 1 ml of

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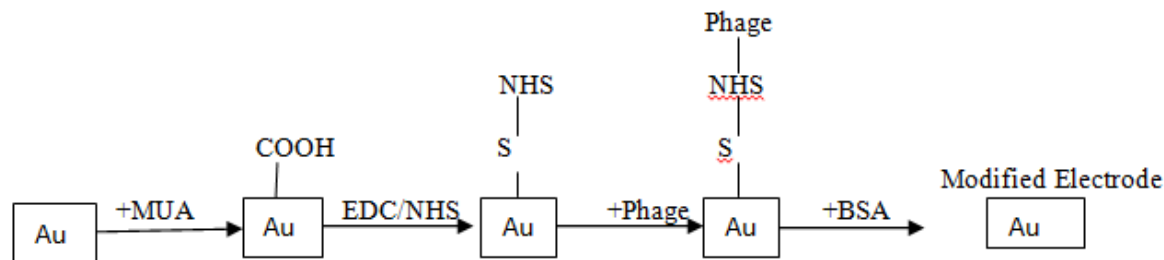


Figure 1. Schematic illustration of the preparation of the phage biosensors.

filtrate was taken and added to tubes containing 5 ml of soft agar (0.7 g/ml, Hi-media) in 100 ml of LB broth). This was mixed and poured onto LB agar plates, solidified and incubated overnight at 37°C. After incubation, presence of plaques was observed at every one hour interval.

Plaque picking, spot test and plaque forming unit

The visible plaques were picked using sterile tips from each soft agar plate (well isolate zone) and transferred to PBS which was then filtered using 0.45 μm filter (Millipore) to remove bacteria, media and cell debris stored at 4°C. Spot test confirmed the presence of phages (Jordan et al., 2011). An overnight grown culture of *E. coli* and top agar was mixed and spread over it. Filter sterilized phages were 10-fold serially diluted using 2-4 μl phosphate buffer saline (PBS) each of the serially dilution of phage suspension being spotted over the plate. Clear zone of lysis were observed after incubation at 37°C and phage density (PFU/ml) of phages was calculated (Jordan et al., 2011).

Electron Microscope

100 ml of phage stock was revived by ultracentrifugation (Beckman Optimal LE-80k UC) at 45000 rpm for 2 h at 4°C. The supernatant was isolated and stored at 4°C and the pellet dissolved in SM buffer (5.8 g NaCl, 2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 50 ml/l of 1M Tris pH 7.5, 5 mL/L of 2% gelatin in distilled Water) (Sambrook et al., 1989).

Phage in SM buffer was negatively stained and preparations were made to examine the structure by Transmission electron microscopy. On the surface of 200 mesh copper grid (100 μl) of sample was applied and then viewed under microscope negatively operating at 120 kV. The stained grids were viewed and photographs were taken at 19000X, and 29000X magnification, visualizing phage tail and capsid for determining the phage's cluster.

Modification of electrode

Immobilization of bacteriophage was based on the formation of SAM-Au surface (Figure 1). Au was immersed in 1 mM ethanolic solution of 11-mercaptoundecanoic acid (MUA) for 16 to 20 h at room temperature. Washing of electrode was performed using deionized water (DI) to remove any unattached species and the dried under nitrogen stream. Activation of gold surface was carried out in aqueous solution of (0.1 g/ml ddH_2O) 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide (EDC) (Hi Media) and (11.5 mg/ml ddH_2O) N-hydroxysuccinimide (NHS) (Sigma) for 1-2 h at room temperature. Again washing was done using deionized water (DI) and dried in steam of nitrogen. Incubation of Gold

surface immersed deep in 1 ml of isolated bacteriophage was done at room temperature for 1-2 h. Washing of electrode surface was carried out 3 times with PBS buffer (Hi Media) (pH 7.4), then with 10 μl of Bovine serum albumin (BSA) (Sigma-Aldrich) solution (1%) and incubation done for 20 min to prevent non-specific adsorption of non-targeted bio-components (Lucarelli et al., 2005; Geng et al., 2008; Tolba et al., 2010; Naidoo et al., 2012; Tiili et al., 2013).

Scanning Electron Microscope and FTIR

SEM images was taken at different gold surface condition for bare gold surface, gold substrates was modified with MUA/EDC/NHS and then 20 μl of Phage (10^7 pfu/mL) was placed on the modified surface air-dried for 12 min. The images were obtained with the SEM instrument (Hitachi S-3400N, Japan) (Shabani et al., 2008). FTIR spectrum taking using model Perkin Elmer (spectrum 400) under 400 to 4000 wavelength of Gold surface (Rawson et al., 1989).

Electrochemical apparatus

Electrochemical measurements were performed at room temperature in a voltammetry cell with three-electrodes, electrochemical cell configuration with a Au disc (1cm dia) working electrode, a platinum wire as the counter electrode, and an Ag/AgCl (filled with 3M KCl) reference electrode. We used an (AUTOLAB PGSTAT 302N, Netherland) cyclic voltammetry and FRA impedance analyzer equipped with the NOVA 1.10 acquisition software. Cyclic voltammetry (CV) was carried out using same electrodes with potential scanned from -1.0 to 1.0 V at a scan rate of 100mV/s in order to determine the midpoint between the oxidation and the reduction of the redox couple, which can be used as an applied DC potential for further impedance measurements. The impedance was obtained in a frequency range from 100 mHz to 100 kHz, using a modulation voltage 10 mV complex plane diagram (Nyquist plot) with a sampling rate of 5 points per decade. The CV and impedance measurements were performed in PBS buffer (pH 7.4) in the presence of 10 mM of $\text{Fe}(\text{CN})_6^{3-/4-}$ Standard (Rawson et al., 1989; Busalmen et al., 2008; Geng et al., 2008; Baldrich et al., 2008; Shabani et al., 2007).

RESULTS AND DISCUSSION

Isolation of bacteriophage

Bacteriophages specific for *E. coli* were isolated from stagnant waste water sample from food industry, Haryana as evident by clear zones of plaque (Figure 2).

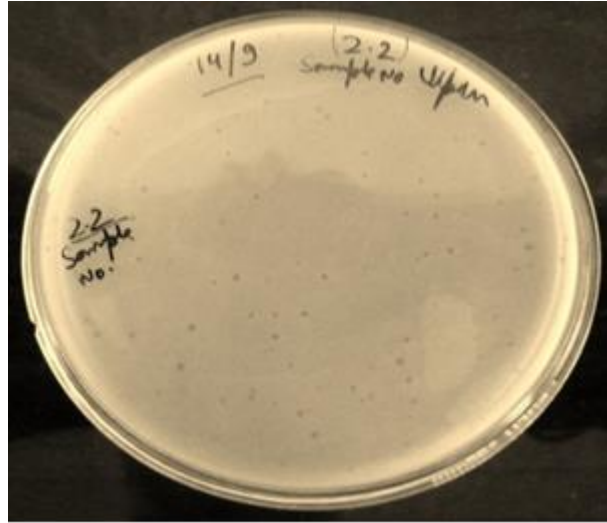


Figure 2. Plaque formation against *E.coli* by agar overlay method.

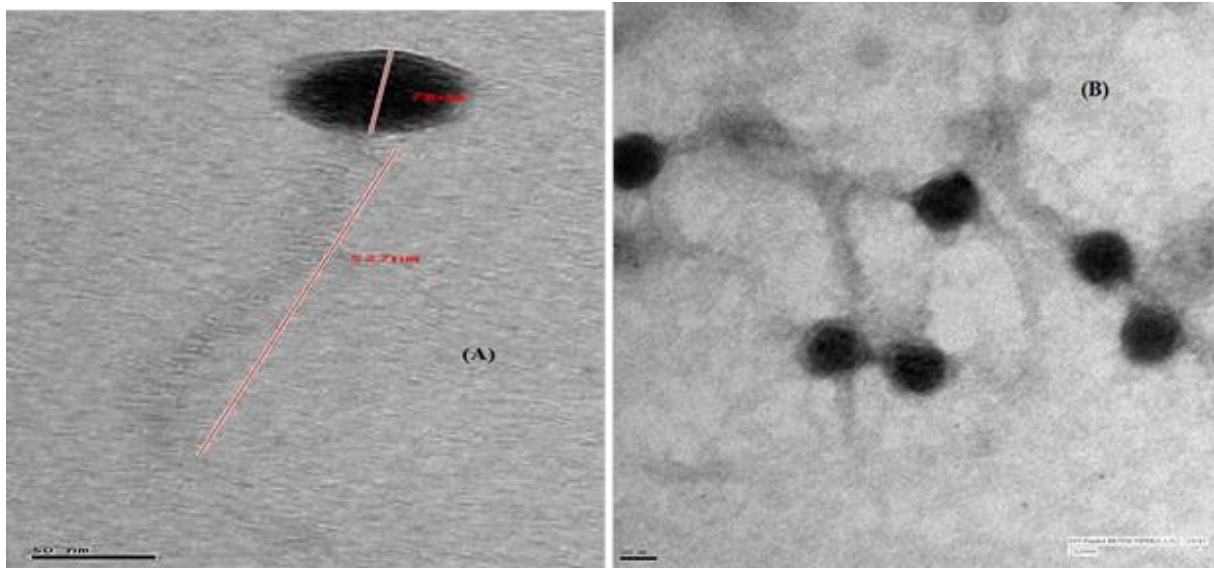


Figure 3. Electron micrographs of isolated bacteriophage stained with uranyl acetate (at (A) x29000 and (B) x19 000).

Plaques appeared as clear zones found in confluent bacterial growth by agar overlay method. Turbidity signifies the presence of lytic phage. Plaque forming unit (PFU) was calculated by counting number of infected virus particles per unit volume (10^7 PFU/ml).

Phage morphology and taxonomic classification

Morphology and identification of family of phage was done using TEM by staining with Uranyl Acetate (Figure 3A and B). The phage head or capsid was about 78 nanometers (nm) in diameter with long tail of length 527

nm. Isolated phage was classified into order *Caudovirales* and as it possesses a long non-contractile tail and icosahedral capsid head, thus is a member of the family *Siphoviridae*. In the present investigation it was observed that the isolated phage seemed similar to lambda like virus phage, lambda phages specific also called a enterobacteria phage λ that is *E. coli* (Hulo et al., 2011).

Gold surface characterization by SEM

The SEM images shows the surface modification by

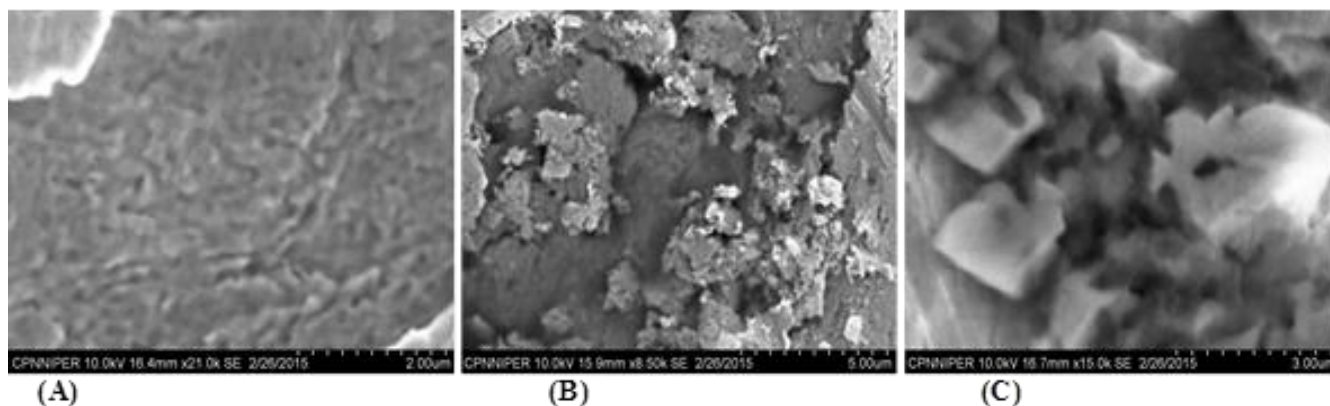


Figure 4. SEM images of phage-modified gold surface. **(A)** Bare gold surface. **(B)** Modified gold surface MUA/EDC/NHS (low resolution). **(C)** Immobilized phage on modified gold surface (low resolution).

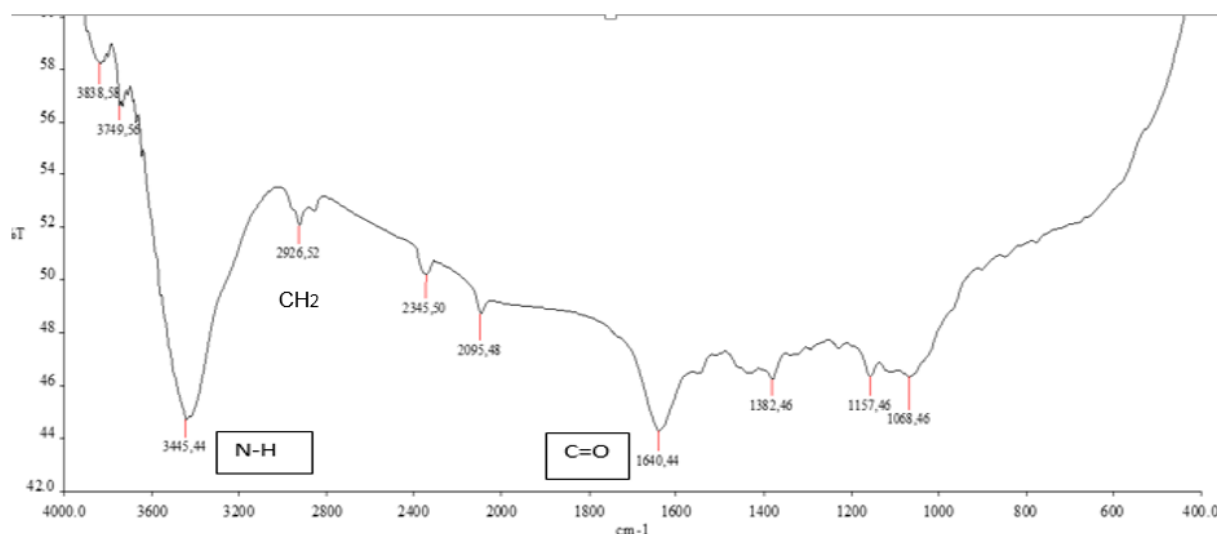


Figure 5. Fourier Transform Infrared Spectrum of the Au/MUA/EDC-NHS electrode assembly. N-H, C=O and CH₂ peaks.

self-assembly monolayer on Gold surface (Figure 4A to C). Compared with image Figure 4A, the bare gold surface was observed. Figure 4B indicates that modified gold surface was successfully linked to the Au-SAM surface. Figure 4C are aggregates of the bacteriophage over the Au-SAM surface (Shabani et al., 2008; Garcia-Gonzalez et al., 2008; Chai et al., 2013).

Surface characterization of Gold surface modified electrode using FTIR

Figure 5 confirms the presence of the Functional group over the Gold surface, a fourier Transform Infrared (FTIR) analysis. Characteristic bond found in such as, N-H, C=O and CH₂, stretching vibrations were at 3445.4 cm⁻¹,

1640.4 cm⁻¹ and 2926 cm⁻¹ wavelength respectively.

Cyclic voltammetry

Cyclic voltammetry is an analytical method used for rapid surface modification and initial characterization. The reversible redox couple that is 1 mM Fe(CN)₆^{3-/4-} in deionized water, was selected as a redox probe to study the characteristics of the Au electrode. Figure 6b shows the cyclic voltamograms of the Au electrode in Fe(CN)₆^{3-/4-} solution after different modification steps. Fe(CN)₆^{3-/4-} showed a reversible effect on Au electrode with peak-to-peak separation of 100 mV/s. The self-assembly of the MUA/EDC/NHS and activation of COOH end groups are supplemented by a decrease in the peak current and

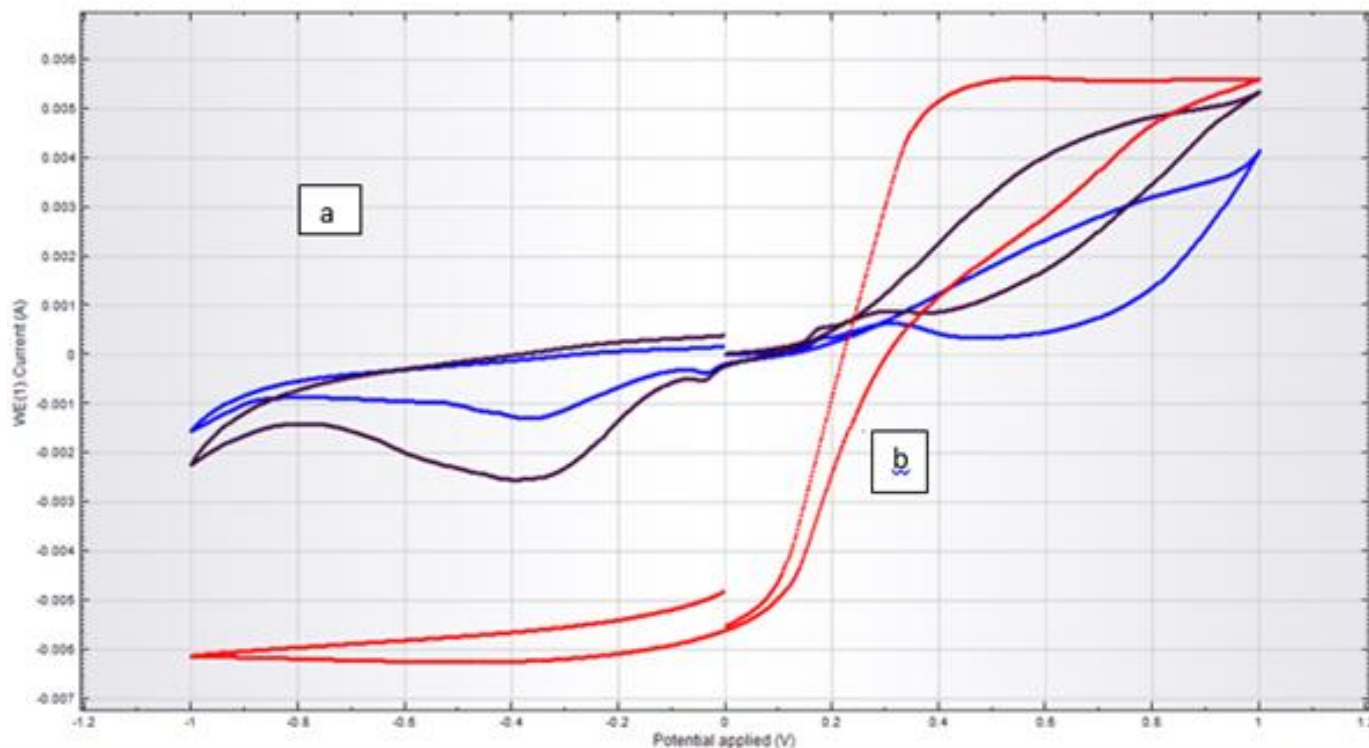


Figure 6. a) Black and Blue curve line are cyclic voltammetry of gold electrode modified with attached phage, different bacterial concentration of *E. coli*. (10^3 and 10^4 cfu/ml) recorded in Phosphate buffer saline. **b)** Red curve line, 1 mm potassium ferric cyanite, as redox active at scan rate of 100 mv/s.

resistance increases due to modification in covering of the electrode at different dilution (10^3 and 10^4 cfu/ml). Figure 6a, depicts the cyclic voltammetry curve of Au working electrode, whose details have been reported in the literature (Lucarelli et al., 2005; Tlili et al., 2013).

Cyclic voltammetry of modified electrode surface scanned at a potential ranging from -1.0 to 1.0 V, at a scan rate of 100mV/s. The formal potential of 5.30×10^{-3} V was estimated from the mean of the anodic and cathodic peaks of the cyclic voltammogram of the bare Au electrode. Figure 6b shows curve line 1mm potassium ferric cyanite, as redox active at scan rate of 100mV/s. Figure 6a shows the decrease in peak currents can be attributed to the fact that the bacteriophage & MUA/EDC/NHS insulated the surface & effectively altered the electron transfer barriers. One of the reasons being the formation of a barrier by the assembled layer that prevents the access of redox couple. Another might be the low current involved in the redox reaction after functionalization of the Au surface with MUA (Geng et al., 2008).

Electrochemical impedance studies

The impedance spectra displayed a semi-circle and a

linear portion. The former at higher frequency represents the electron-transfer limited process and the latter at lower frequency represents the diffusion-limited process (Tlili et al., 2013). Phage modified microelectrode in the presence of *E. coli* was placed in PBS solution with the frequency range between 100 MHz-100 KHz (Mejri et al., 2011; Chang Qing et al., 2012). Impedance analysis detection of *E. coli* in apparatus cell, containing sterile PBS, was conducted with different bacterial concentration varying from 10^3 and 10^4 CFU/ml. Nyquist plot shows a gradual increase in impedance initially that continued for about 4-15 minutes (Figure 7). The impedance Z'' (Ω) increased slowly over time until it stabilizes for 10^3 (800) and 10^4 (3.2×10^3) cfu/ml, also real impedance Z' (Ω) for both cfu level is 1500 and 3000 increase when compared to bare gold. The semi-circle corresponds to the charge-transfer resistance of the electrode interface (Mejri et al., 2011). The decrease in charge transfer resistance is because of the increase in conductivity after bacteriophage immobilization. This happens because of specific recognition between bacteria and bacteriophage. The subsequent decrease in impedance supposedly occurs as a direct consequence of phage-induced bacteria infection, leading to cell wall disruption and secretion of important amount of intracellular components (Shabani et al., 2008). The medium conductivity in the

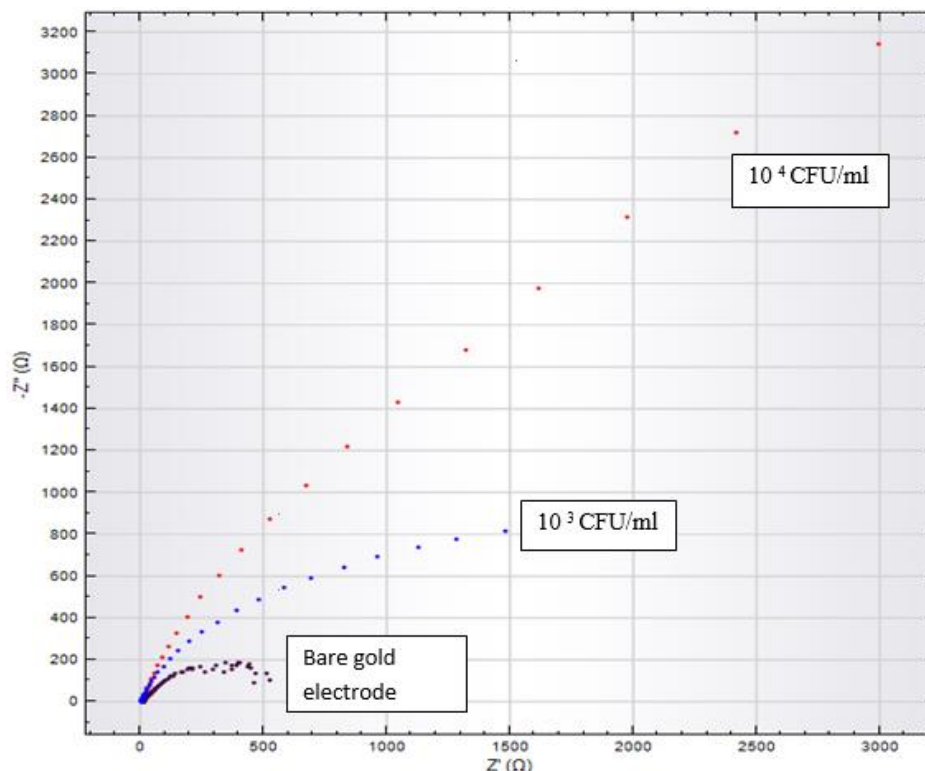


Figure 7. Nyquist plots of impedance spectras obtained for increasing concentrations of *e.coli* from 10^3 (blue dot curve) cfu/ml and 10^4 cfu/ml (Red dot curve) in phosphate buffered saline (pbs) Au electrode with phage immobilized.

vicinity of the electrode surface is increased as a direct consequence of these events. This increase in medium conductivity decreases the charge transfer resistance (Barreiros dos Santos et al., 2009; Lua et al., 2008).

Conclusion

In the present study, bacteriophage isolated from stagnant waste water sources against *E. coli* and confirmed it to be a lambda like virus after morphological and structural characterization by transmission electron microscopy. Its lytic phage, Plaque reaction activity appear within 4-6 h by spot test method. The bacteriophage then used as a bio component for developing a detection based platform. Isolate phage immobilization was realized through the widely used EDC/NHS cross-linking reaction, which connected the $-NH_2$ group on the surface of phage to the carboxyl end group of MUA self-assembled monolayer on the gold (Au) surface conform by Fourier transformation infrared microscopy presence of functional groups present. Electrochemical measurements including CV and Impedance and Scanning Electron Microscopy observation were carried out to characterize the surface modified gold electrode. The Scanning Electron

Microscopy verified that the phage efficiently immobilized onto the Au-MUA/EDC/NHS self-assembled monolayer. CV and Impedance result also showed the electron transfer resistance of the phage-modified electrode. A liner relationship between the electron-transfer resistance and the logarithmic value of *E. coli* concentration was found in the a range of 10^3 and 10^4 cfu/ml with a detection limit of 10^4 cfu/ml. the phage based detection technique introduced here is a real-time, rapid, specific and quantitative method. For future work, generic platform for advanced bacterial sensing, with a high promise in practical applications.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Evaluation of antibacterial activities of *Barleria Prionitis* Linn

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In the present study, 1 g/ml each of *Barleria prionitis* leaves (BPL) and *B. prionitis* stem (BPS) were extracted from different solvents like petroleum ether, chloroform ethyl acetate and methanol. Ethyl acetate extract of BPL showed maximum inhibition zone on Gram positive *Bacillus pumilus* (9.83 mm) and methanol extract of BPS showed minimum inhibition zone on Gram negative *Escherichia coli* (0.16 mm). Petroleum ether extract did not show inhibition except petroleum ether extract of BPS on Gram positive *B. pumilus* (0.46 mm). Minimum inhibition concentration (MIC) was shown by petroleum ether extract of BPL on Gram positive *B. pumilus* and Gram negative *Pseudomonas aeruginosa* (1.0 mg/ml). Leaves and stem extract of *Barleria prionitis* L. showed difference in antibacterial activity.

Key words: Plant extracts, antimicrobial activity, minimum inhibitory concentration.

INTRODUCTION

Medicinal plants have played an essential role in the development of human culture, for example religions and different ceremonies. Also, they are important sources of unknown chemical substances with potential therapeutic effects. Many drugs presently prescribed by physicians are either directly isolated from plants or are artificially modified versions of natural products (Wang et al., 2007).

In recent time, traditional medicines derived from herbs have huge therapeutic potential to heal many infectious diseases without associated side effects compared to synthetic drugs. In indigenous system of medicine in India, the aerial parts of *Barleria prionitis* are used for the treatment of anemia, toothache, and bacterial disorders.

In the present work, an attempt has been made to study the efficacy of non-polar compounds fractionated from *B. prionitis*; they were evaluated for their anti-bacterial activity and compared with the efficacy of polar compounds fractionated from the same plant. Leaves of *B. prionitis* were extracted from petroleum ether containing non-polar compounds followed by ethanol extract containing the polar compounds of *B. prionitis* (Aiswarya and Ravikumar, 2014).

Over the past few years, medicinal plants have regained a wide recognition due to an escalating faith in herbal medicines, whose side effects are lesser compared to allopathic medicines. And they also meet

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Figure 1. Selected different parts of barleria prionitis L. plant.

the necessary requirements of medicine for an increasing human population. With the continuous erosion of traditional knowledge of plants used for medicine in the past and the renewed interest at the present time, there is a need to review this valuable knowledge of medicinal plants with the purpose of developing medicinal plants sectors across the different states in India.

In fact, it is well known that even in developed countries, the use of traditional medicines is quite prevalent. This culture has been passed down from one generation to another (Nurliani et al., 2004); hence, promoting the use of medicinal plants for health purpose. In India alone, less than 10% of the medicinal plants traded in the country are cultivated, about 90% are collected from the wild, very often in a destructive and unsustainable manner (Natesh, 2000).

Natural products have been applied to human healthcare for thousands of years. According to the World Health Organization (WHO) estimates, out of 4 billion people, 80 percent of the world populations presently use herbal medicine for some aspects of primary health care (Behera, 2006). WHO notes that of 119 plant-derived pharmaceutical medicines, about 74 percent are used in modern medicine directly proportional to the traditional use of plant medicines by native cultures (WHO, 2002).

MATERIALS AND METHODS

Plant sample

The plant selected in the present study was *B. prionitis* L. This plant was collected from Boriavi, Anand, Gujarat, India (Figure 1). Throughout the study, specific codes were allotted to various parts of the tested plant material: BPL for *B. prionitis* leaves and BPS for *B. prionitis* stem. Upper and lower ground parts of the tested plant serve as sources of young leaf and stem tissue used during the antibacterial activity, minimum inhibitory concentration, phytochemical test and antioxidant studies.

Preparation of plant extracts

For preparing the various parts of the plant extract, leaves and stems of *B. prionitis* L. were air dried at room temperature (27°C) for one week, after which they were ground into a uniform powder. The following four different solvent extracts were used for preparing the plant extracts: petroleum ether, chloroform, ethyl acetate and methanol. 10 g each of the dry powdered plant material was soaked in 200 ml of respective solvent at their respective boiling temperature for 48 h in Soxhlet assembly. The extracts were filtered after 48 h, first through a Whatman filter paper No. 42 (125 mm) and then through cotton wool. The filtrates obtained were concentrated under vacuum on a rotary evaporator at 40°C. The extracts were kept in sterile bottles under refrigerated condition until use. Then 10 gm of dry extracts was resuspended in 10 ml of respective solvents. The final concentration of the plants extracts was 1 g/ml.

Microorganisms used

The test organisms used for screening the antibacterial activity of the plant extracts were both Gram positive (*Bacillus subtilis*, *Bacillus pumilus* MTCC 7615, *Bacillus cereus* and *Streptococcus pyogenes* MTCC 1923) and Gram negative bacteria (*Escherichia coli* MTCC 448, *Pseudomonas aeruginosa* MTCC 7436, *Comomonas acidovorans* MTCC 3362 and *Serratia marcescens* MTCC 3124).

Preparation of culture media

All bacterial strains were cultivated in nutrient agar medium (NA), and incubated at 37°C for 24 h. These were used for the microbial activity by disc diffusion assay

Testing for antibacterial activity

Crude plants extracted from different solvents (petroleum ether, chloroform, ethyl acetate and methanol) were used to determine the antibacterial activity by cup-plate agar diffusion method, which was slightly modified, according to Kirby-Bauer. It was used to assess the antibacterial activity of the prepared extracts. 20 ml of the inoculated nutrient agar and 0.5 ml of standardized bacterial stock suspensions (10⁸-10⁹ colony-forming units per ml) were poured on sterile Petri plate. Negative controls were prepared using the same solvents which were employed to dissolve the plant extracts. TE30 (tetracycline 30 mcg/disc), GEN10 (gentamicin 10 mcg/disc), E15 (erythromycin 15 mcg/disc), S10 (streptomycin 10 mcg/disc) were used as positive reference standards to determine the sensitivity of one strain in each bacterial species tested.

Four wells of 9 mm diameter were bored in the medium with the help of sterile cork-borer having 9 mm diameter. They were labeled properly. Fifty microliters of the working suspension/solution of the different extracts of the plant's parts and same volume of solvent extract for control were filled in the wells with the help of micropipette. They were allowed to diffuse at room temperature for two hours. The plates were then incubated in an upright position at 37°C for 18 h. Three replicates were carried out for each extract against each of the test organism. Simultaneous addition of the respective solvents instead of extracts was carried out as controls. After incubation, the diameters of the results and growth inhibition zones were measured averaged and the mean values were tabulated.

Determination of minimum inhibitory concentration

Although the results of the disc diffusion assay cannot always be compared to the MIC data (Njenga et al., 2005), plant extracts having positive antibacterial activity against most of the microorganisms tested in the disc diffusion bioassay were further tested for the determination of minimum inhibitory concentration (MIC) by two fold serial broth dilution method. Plant extracts with more than 4.5 mm inhibition zone were selected for MIC. Selective broth medium was used for dilutions as well as preparing inoculums. The bacterial cell density was maintained uniformly throughout the experimentation at 1 x 10⁸ CFU/ml by comparing with 0.5 McFarland turbidity standards. Plant extract of 40 µl from stock solution (100 mg/ml) was put in the first dilution tube containing 960 µl of selective medium broth and mixed well. From this, 500 µl well was transferred to second tubes containing 500 µl broths. This step was repeated nine times and from last tube 500 µl solution was discarded. 100 µl of test organism was added in each tube. The final volume of the solution in each tube was made up to 0.6 ml. Tubes were incubated at optimal temperature and time in an

incubator. Growth indicator 2-3-5 tri phenyl tetrazolium chloride solution (100 µl of 0.1 %) was incorporated in each tube to find out the bacterial growth inhibition. Tubes were further incubated for 30 min under dark condition. Bacterial growth was visualized when colorless 2-3-5 tri phenyl tetrazolium chloride was converted to red color from zone in the presence of bacteria.

RESULTS

Antibacterial activity against Gram positive bacteria

In the present finding, different solvent extracts from leaves and stem (BPL and BPS) parts of *Barleria prionitis* L showed antibacterial activity against all Gram positive bacteria studied (*B. pumilus*, *B. subtilis*, *Streptococcus pyogenes* and *Bacillus cereus*) (Figures 2, 3, 4 and 5).

The control plate representing petroleum ether, ethyl acetate, chloroform and methanolic extracts did not inhibit growth activity of the tested Gram positive microorganisms (Figures 2, 3, 4 and 5). For standard antibiotics, discs zone of growth inhibition was noted against selected strains of Gram positive bacteria (Figures 2, 3, 4 and 5). These results were compared with the response of the plant's extracts.

Antibacterial activity against Gram negative bacteria

In the present finding, the result revealed that the different solvent extracts of leaves and stem parts of *Barleria prionitis* L. also showed antibacterial activity against Gram negative bacteria (*E. coli*, *Serratia marcescens*, *Comomonas acidovorans* and *P. aeruginosa*) (Figures 6, 7, 8 and 9).

The control plate representing petroleum ether, ethyl acetate, chloroform and methanolic extracts did not inhibit the tested Gram negative microorganisms (Figures 6, 7, 8 and 9). For standard antibiotics, discs zone of inhibition was noted against selected strains of Gram negative bacteria (Figures 6, 7, 8 and 9). These results were compared with the plant's extracts zone of inhibition.

Minimum inhibitory concentration (MIC)

Ethyl acetate extract of leaves showed maximum MIC against *B. pumilus* and *P. aeruginosa*. It required minimum concentration of 1.0 mg/ml which was sufficient to inhibit the growth activity.

DISCUSSION

Numerous antibacterial screening has been performed with respect to location of microorganism such as skin infections, uterine infections etc. As oral bacterial infections are linked with various chronic diseases, screening of antibacterial activity of medicinally important

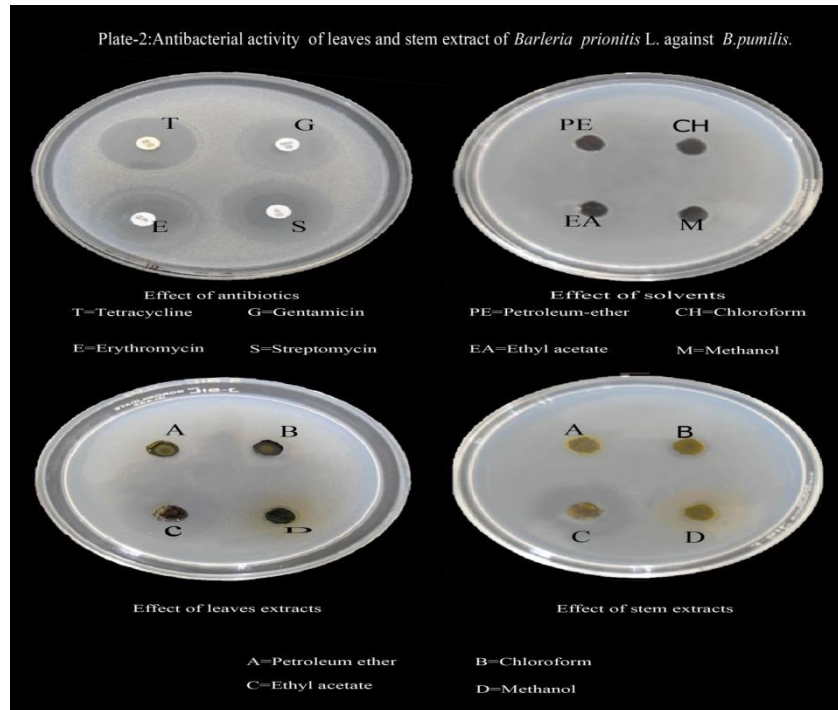


Figure 2. Antibacterial activity of leaves and stem extract of *Barleria prionitis* L. against *B. pumilus*.

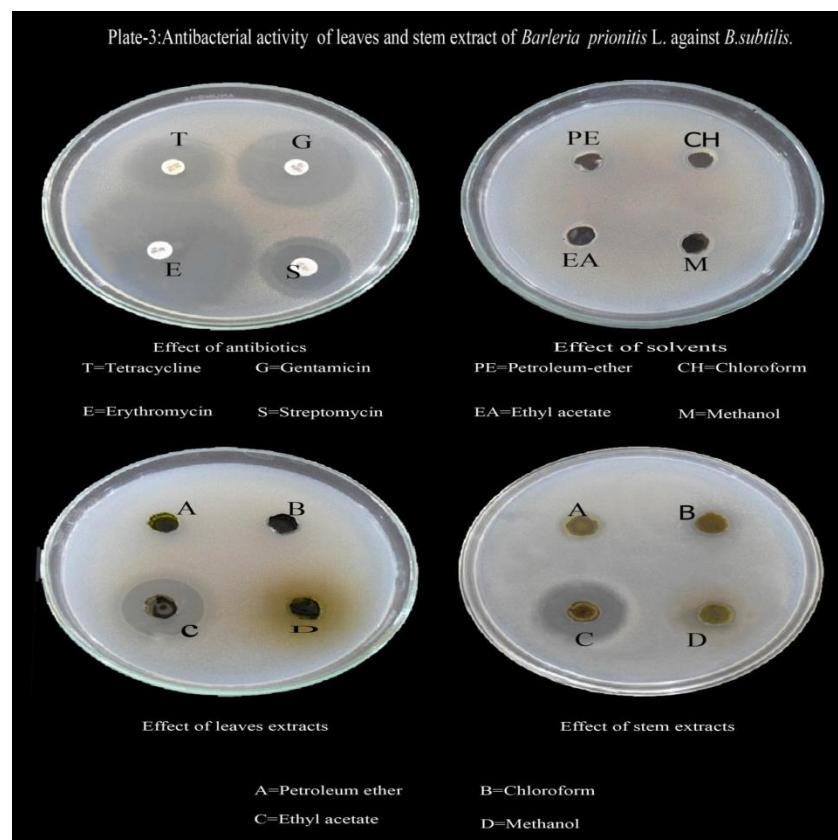


Figure 3. Antibacterial activity of leaves and stem extract of *Barleria prionitis* L. against *B. pumilus*.

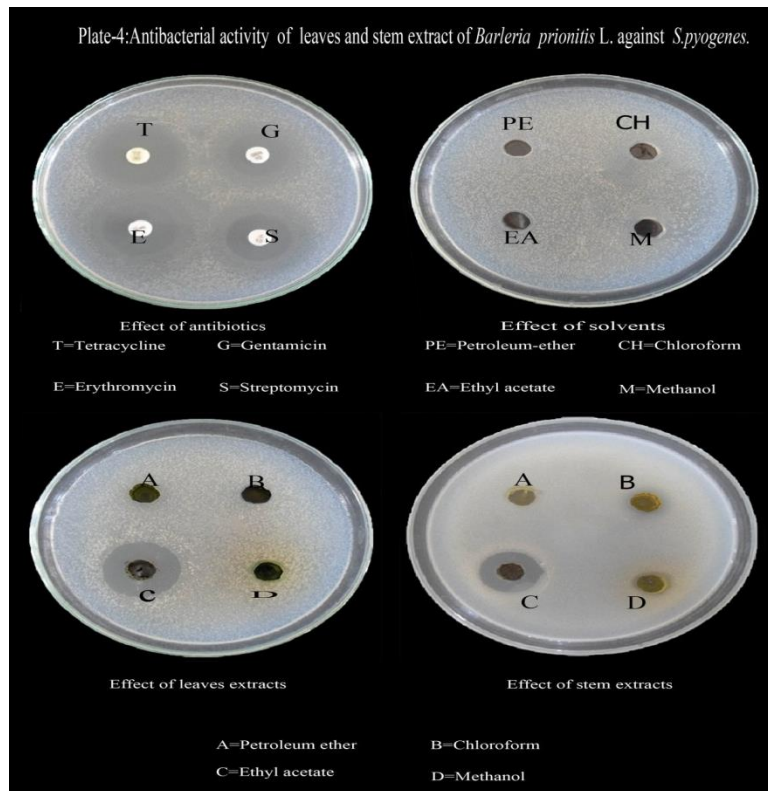


Figure 4. Antibacterial activity of leaves and stem extract of *Barleria prionitis* L. against *S. pyogenes subtilis*.



Figure 5. Antibacterial activity of leaves and stem extract of *Barleria prionitis* L. against *B. cereus*.

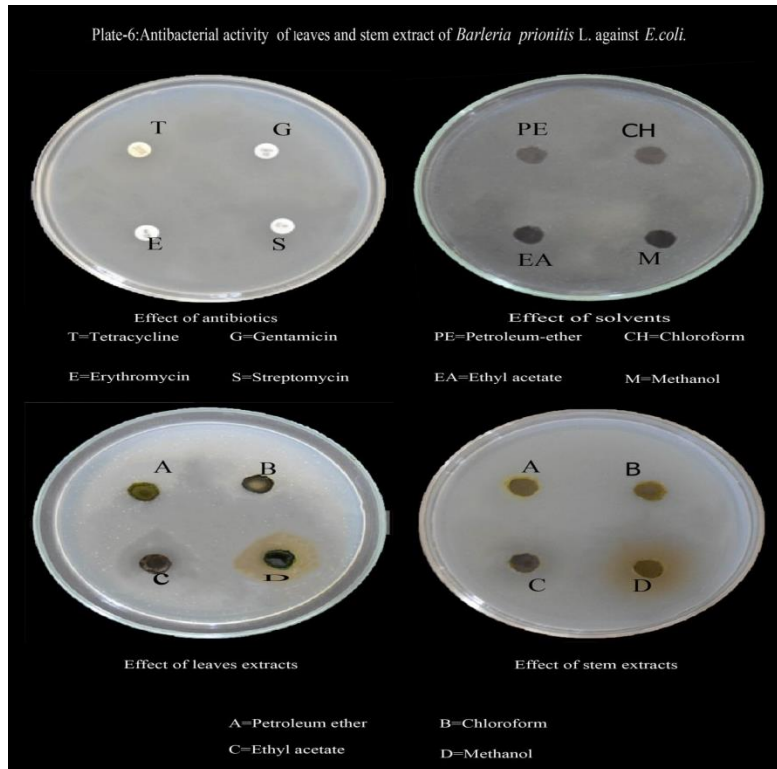


Figure 6. Antibacterial activity of leaves and stem extract of *Barleria prionitis* L. against *E. coli*.

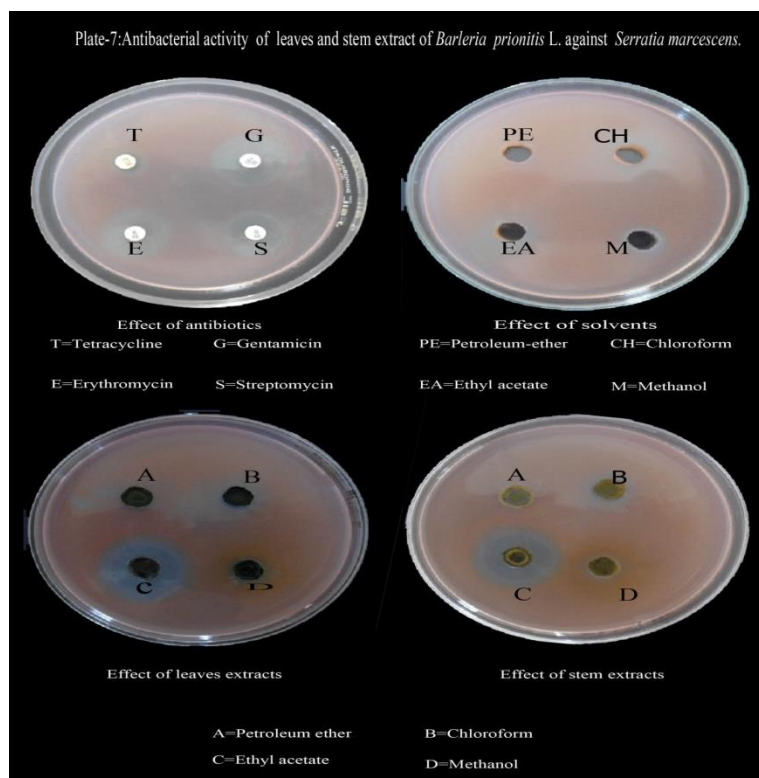


Figure 7. Antibacterial activity of leaves and stem extract of *Barleria prionitis* L. against *Serratia marcescens*

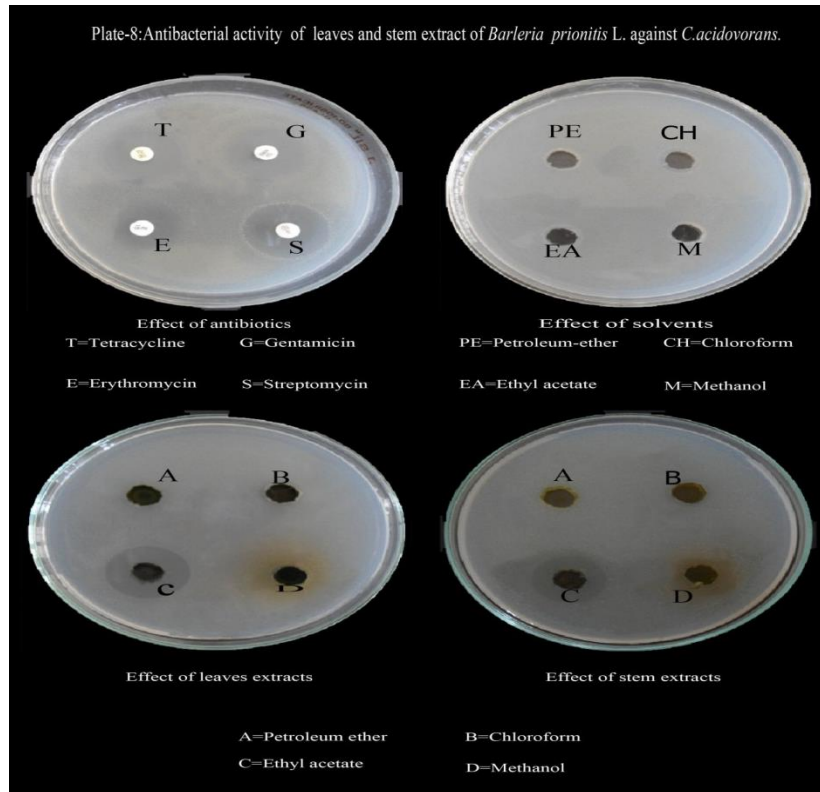


Figure 8. Antibacterial activity of leaves and stem extract of *Barleria prionitis* L. against *Comomonas acidovorans*.

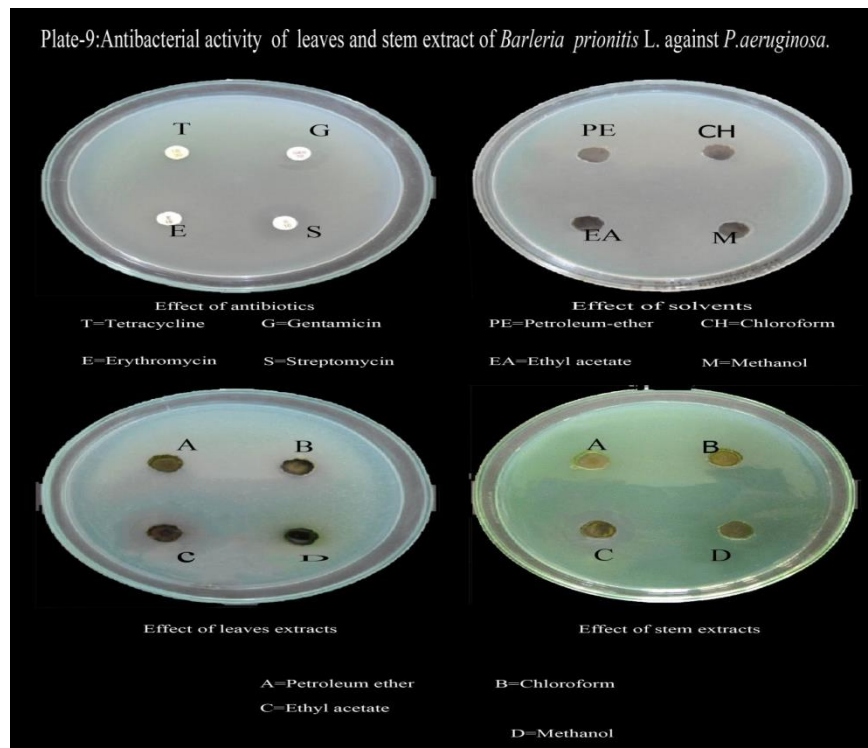


Figure 9. Antibacterial activity of leaves and stem extract of *Barleria prionitis* L. against *Pseudomonas aeruginosa*.

Table 1. Antibacterial activity of leaves and stem extracts of *Barleria prionitis* L. and antimicrobial agents.

Tested microorganisms	Diameter of inhibition zone (mm)												
	Petroleum ether extract		Chloroform extract		Ethyl acetate extract		Methanol extract		Microbial agent				
	Leaves	Stem	Leaves	Stem	Leaves	Stem	Leaves	Stem	TE ³⁰	GEN ¹⁰	E ¹⁵	S ¹⁰	
Gram positive bacteria	<i>Bacillus pumilus</i>	-	0.46	1.83	1.16	9.83	8.08	1.16	1.33	10.0	4.50	8.10	4.50
	<i>Bacillus subtilis</i>	-	-	1.00	0.91	6.25	6.00	0.50	1.00	8.00	5.50	6.00	6.00
	<i>Streptococcus pyogenes</i>	-	-	-	1.16	7.00	5.16	-	0.33	7.83	4.50	7.33	6.00
	<i>Bacillus cereus</i>	-	-	0.665	1.50	6.16	6.50	0.83	-	7.00	4.50	7.00	5.66
	<i>Escherichia coli</i>	-	-	-	0.50	3.00	3.66	-	0.16	1.41	-	-	-
Gram negative bacteria	<i>Serratia marcescens</i>	-	-	1.33	1.00	5.16	4.91	-	-	1.50	4.00	-	4.66
	<i>Comomonas acidovorans</i>	-	-	1.00	1.50	6.00	5.00	-	1.08	8.25	6.00	4.00	5.00
	<i>Pseudomonas aeruginosa</i>	-	-	1.33	3.08	4.75	5.58	-	2.33	-	5.00	-	3.08

Key: (-) = No inhibition zone, TE³⁰ = Tetracycline 30 mcg/disc, E¹⁵ = Erythromycin 15 mcg/disc, GEN¹⁰ = Gentamicin 10 mcg/disc, S¹⁰ = Streptomycin 10 mcg/disc.

plant against bacteria found in oral cavity is also important. Various plants have been evaluated for their efficacy in oral hygiene. Deshpande et al. (2011) evaluated antibacterial activity of different extracts of *J. regia* against oral micro flora and found that acetone extract was more effective against oral microflora. Similar results were observed in the present investigation, where chloroform extract of *Barleria prionitis* L. leaves was more effective. Ogundiya et al. (2009) carried out antimicrobial activity of acetone and ethanol extract of stem and root of *Terminalia glaucescens* and reported that ethanol extract had significantly higher effect. Antibacterial potential of aqueous decoction of *Piper nigrum* L., *Laurus nobilis* L., *Pimpinella anisum* L. and *Coriandum sativum* L. against 176 bacteria isolated from oral cavity of 200 individuals was carried out by Nazia and Perween (2006). More et al. (2008) studied ethanol extract of eight plant species used traditionally in South Africa against oral pathogens such as *Actinomyces* and *Candida* species and got resistance activity.

Antibacterial activity against six bacteria (*B. cereus* (MTTT 430), *B. licheniformis* (MTCC 1483), and *S. aureus* (*E. coli*, *S. typhi*, *A. faecalis*) was carried out. Methanol extract of leaf showed highest antibacterial activity against *B. cereus* (22.66 mm in diameter) followed by pet ether leaf extract against *E. coli* (21.66 mm in diameter). Various extracts of *B. prionitis* were comparable to control antibacterial agents, Ampicillin, Tetracycline. Maximum inhibition was shown by tetracycline against *S. aureus* (28.20) followed by Ampicillin against *B. cereus* (28.40). Resazurin 96 well assay was used to assess minimum inhibition concentration (MIC); petroleum ether of leaf demonstrated the

least MIC value against *B. cereus* (0.05 mg/ml) and *E. coli* (0.2 mg/ml), while the methanol extract of bark and leaf demonstrated 0.2 mg/ml against *B. cereus* (Kumar et al., 2013).

In this study, leaves extract of ethyl acetate showed maximum potential of antibacterial activity against *B. pumilus* (9.83 mm zone diameter of inhibition) while minimum activity was observed against *Bacillus subtilis* (0.50 mm zone diameter of inhibition) in (Table 1).

Stem extract of ethyl acetate showed maximum potential of antibacterial activity against *B. pumilus* (8.80 mm zone diameter of inhibition) and lowest activity was observed in methanol extract against *Streptococcus pyogenes* (0.33 mm zone diameter of inhibition) in (Table 1).

Leaves extract of ethyl acetate showed maximum potential of antibacterial activity against *Comomonas acidovorans* (6.00 mm zone diameter of inhibition) and chloroform extract showed minimum inhibitory activity against *Comomonas acidovorans* (1.00 mm zone diameter of inhibition) in (Table 1).

Ethyl acetate extract of stem showed maximum potential of antibacterial activity against *Pseudomonas aeruginosa* (5.58 mm zone diameter of inhibition) and methanol extract showed minimum activity against *E. coli* (0.16 mm zone diameter of inhibition) in (Table 1).

Minimum inhibitory concentration (MIC) of ethyl acetate extract

In the present finding, leaves and stem part of *Barleria prionitis* L. (BPL & BPS) showed MIC activity against selected strains of Gram positive bacteria (*Bacillus*

Table 2. Minimum inhibitory concentration (MIC) of *Barleria prionitis* L.

Tested microorganisms		Minimum inhibitory concentration (MIC) mg/ml	
		Ethyl acetate extract	
		Leaves	Stem
Gram positive bacteria	<i>Bacillus pumilus</i>	1.0	4.0
	<i>Bacillus subtilis</i>	4.0	4.0
	<i>Streptococcus pyogenes</i>	2.0	4.0
	<i>Bacillus cereus</i>	2.0	4.0
Gram negative bacteria	<i>Escherichia coli</i>	-	-
	<i>Serratia marcescens</i>	2.0	4.0
	<i>Comomonas acidovorans</i>	2.0	4.0
	<i>Pseudomonas aeruginosa</i>	1.0	2.0

pumilus, *Bacillus subtilis*, *Streptococcus pyogenes* and *Bacillus cereus*). The result in Table 2 shows that the ethyl acetate extract of leaves showed maximum MIC against *B. pumilus*. It required minimum concentration of 1.0 mg/ml which was sufficient to inhibit the growth activity. However, this was followed by *Streptococcus pyogenes* and *B. cereus*, where both required MIC of 2.00 mg/ml. wt of leaves extract to inhibit growth activity. Minimum MIC value was shown by *Bacillus subtilis*, where 4.0 mg/ml. wt leaf extract was required to inhibit the growth activity.

For stem extract, the result shows that all the tested Gram positive bacteria had same value of MIC value (4.0 mg/ml) which was sufficient to inhibit the growth activity.

In the present investigation, leaves and stem part of *Barleria prionitis* L. (BPL and BPS) showed MIC activity against selected strains of Gram negative bacteria (*Serratia marcescens*, *Comomonas acidovorans* and *Pseudomonas aeruginosa*). Result revealed that the MIC value of leaves extract was found in the range of 1.00 mg/ml to 2.00 mg/ml. However, maximum result was found against *Pseudomonas aeruginosa*, where it is required the minimum concentration of 1.00 mg/ml of leaves extract, which was sufficient to inhibit the growth activity. However, *Serratia marcescens* and *Comomonas acidovorans* required same minimum concentration of 2.00 mg/ml of leaves extract, which was sufficient to inhibit the growth activity (Table 2).

In case of stem extract, the result reported 2.00 mg/ml to 4.00 mg/ml. Maximum result was noted against *Pseudomonas aeruginosa*, where it is required the minimum concentration of 2.00 mg/ml of stem extract which was sufficient to inhibit the growth activity. However, *Serratia marcescens* and *Comomonas acidovorans* required the same amount of minimum value of 4.00 mg/ml of stem extract to inhibit the growth activity (Table 2).

Conclusion

We concluded from the present study that leaves and

stem extract of *Barleria prionitis* L. showed difference in antibacterial activity

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Comparison of two storage conditions of *Candida albicans* for DNA extraction and analysis

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The major human fungal pathogens are species from the genus *Candida*, especially *Candida albicans*. *C. albicans* sample preservation from human source is essential for clinical purposes and epidemiologic studies; however, little is known about its conservation for DNA extraction and analysis. Therefore, the aim of this study was to evaluate and compare two storage conditions of *C. albicans* for performing DNA extraction and analysis. We collected samples from the intraoral palatal mucosa of patients with chronic atrophic candidiasis, and then assigned them into two groups for DNA extraction: Sabouraud dextrose agar (SDA) group samples taken from an SDA culture and the sterile buffering solution (SBS) group directly from the inoculum. We took *C. albicans* from samples stored in SBS and kept for two years at -80°C, and performed the DNA extraction with the Purgene DNA extraction kit for buccal cells. The means (standard deviation) for DNA extracted from *C. albicans* in SDA and SBS were, respectively, 16.62 ng/uL (10.53) and 9.732 ng/uL (2.342). In our qualitative evaluation, we observed no differences in band patterns between both treatment groups (SDA and SBS). The results show that SDA and SBS methods could preserve *C. albicans*' DNA for extraction to evaluate quantitative and qualitative data.

Key words: *Candida albicans*, storage, DNA, DNA fungal.

INTRODUCTION

The major human fungal pathogens are species from the genus *Candida*, especially *Candida albicans* (McManus et al., 2014), which are capable of causing a wide variety

of infections despite their presence in the normal flora of humans. *C. albicans* is also the predominant fungi isolated from the human mouth, where it is the most

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prevalent species (Zomorodian et al., 2011). The reported rate of oral *C. albicans* is highly variable and depends on the population and the sampling methods used. Considering a healthy population, 82.6% of subjects carry the *C. albicans* (Mun et al., 2015).

Infections caused by *C. albicans* are characterized as opportunistic, frequently affecting immunocompromised patients, such as those with HIV, undergoing chemotherapy treatment, or intensive immunosuppressive treatment regimens (Berberi et al., 2015). However, the lack of precise tools to detect infections by this fungus has often led to inappropriate therapies or delays in their initiation (Fredricks et al., 2005; Gurbuz et al., 2010; Loeffler et al., 2002).

In recent years, researchers have made efforts to develop molecular-based techniques for rapid detection of *C. albicans*, which would be crucial for the treatment and recovery of patients suffering from candidiasis (Arancia et al., 2009; Cerikçioğlu et al., 2010; Fredricks et al., 2005; Loeffler et al., 2002; Metwally et al., 2008). Also, yeast typing is essential in epidemiology studies for recognizing outbreaks and determining the source of infections, identifying particularly virulent or drug-resistant strains, and detecting possible crossed transmissions (McManus et al., 2014).

Improvements in molecular biology have allowed the use of fingerprinting methods such as the following: karyotyping using pulsed-field gel electrophoresis (PFGE), restriction fragment polymorphism (RFLP), randomly amplified polymorphic DNA analysis (RAPD), and southern hybridization for the identification of *C. albicans*. Semi-nested PCR or quantitative real-time PCR (qPCR) techniques are well disseminated in the literature and have shown positive results in the identification of yeasts in samples (Costa et al., 2010; Gurbuz et al., 2010; Guiver et al., 2001; Khan et al., 2009; Ligozzi et al., 2003; Metwally et al., 2008).

Pure DNA must be available to perform a sensitive, specific, and reliable molecular-based technique for *C. albicans* identification (Mancini et al., 2010). However, in contrast with other eukaryotic tissues or cultured cells, yeasts have outer cell walls, which impair rapid isolation of RNA and genomic DNA (Linke et al., 2010; Maaroufi et al., 2004). Another important issue is the maintenance of microorganisms from clinical samples (Mariano et al., 2007). There are several current protocols to preserve yeasts, such as seeding in renewed agar, storage in a buffer solution, or freezing in glycerol (Mariano et al., 2007; Nedel et al., 2009a).

Although yeast sample preservation from human sources is essential for clinical purposes and epidemiologic studies, little is known about its conservation for DNA extraction and analysis. Therefore, the aim of this study was to evaluate and compare two storage conditions of *C. albicans* for performing DNA extraction and analysis.

MATERIALS AND METHODS

This research was approved by the Dental Ethics Committee at Federal University of Pelotas (036/2006). Clinical samples were collected, according to Lund et al. (2010), from patients with chronic atrophic candidiasis at the Center of Diagnosis of Oral Diseases, at the Federal University of Pelotas. Briefly, samples were collected, from the intraoral palatal mucosa of five patients using disposable sterile swabs. After collection, samples were immediately seeded in Petri dishes with Sabouraud dextrose agar (SDA) and incubated at 37°C for 48 h. After the growth period, each isolated yeast was stored in 1 mL of sterile buffering solution (SBS) for 2 years at -80°C.

After this period samples were thawed. For the SBS group (n=5) the extraction for DNA was conducted directly from the inoculum. For the SDA group (n=5) the same inoculum samples from the SBS group, were seeded in Petri dishes with SDA and incubated at 37°C for 48 h, and then DNA extraction was conducted.

The DNA extraction was performed with the Purgene DNA extraction kit for buccal cells (Purgene DNA Buccal Cell Kit; Gentra Systems, Inc.), according to the manufacturer's instructions. The DNA concentration and purity was evaluated using a Qubit™ fluorometer (Invitrogen). To ascertain the presence of high molecular weight, we examined the DNA samples in 0.8% agarose gel electrophoresis at 2 V/cm and stained them with GelRed™ (Biotium Inc., CA). Two calibrated evaluators who were not otherwise involved in this study examined the visible bands. The collected data was tabulated using SigmaStat 3.5 statistical software and submitted them to analysis of variance (ANOVA).

RESULTS

The means and standard deviations for DNA extracted from *C. albicans* in SDA and when stored in SBS buffer were, respectively, 16.62 (10.53) and 9.732 ng/uL (2.342). There was no statistically significant difference between the different storage methods ($p = 0.191$) (Table 1).

In our qualitative evaluation, we observed no differences in band patterns between both treatment groups (SDA and SBS buffer) in the agarose gel (Figure 1).

DISCUSSION

The extraction of DNA from candidiasis samples in epidemiological studies is a complex procedure and it is difficult to reproduce results (McManus et al., 2014). Sample collection locations frequently do not support DNA extraction, and samples therefore often require transportation. However, this transportation should occur with minimum loss in the quality and quantity of genomic material (Nedel et al., 2009a, 2009b). When the DNA extraction cannot be immediately performed after collection and transportation, storage becomes an alternative. In this regard, studies have demonstrated that the DNA can be extracted after up to 180 days of storage for epidemiological purposes when *C. albicans* are stored in SDA (Bacelo et al., 2009).

In the present work, the total DNA yield extracted from

Table 1. Quantitative DNA analysis from five patients with chronic atrophic candidiasis submitted to two groups: in the SDA group, the DNA was extracted from *C. albicans* samples directly from SDA; in the SBS group, the DNA was extracted from *C. albicans* samples stored in SBS buffer.

Patient	SDA Group (ng/uL)	SBS Group (ng/uL)
1	35.3000	13.5000
2	13.4000	8.6500
3	10.6000	8.1100
4	10.6000	10.5000
5	13.2000	7.9000
Means (SD)	16.62 ng/uL (10.53)	9.732 ng/uL (2.342)

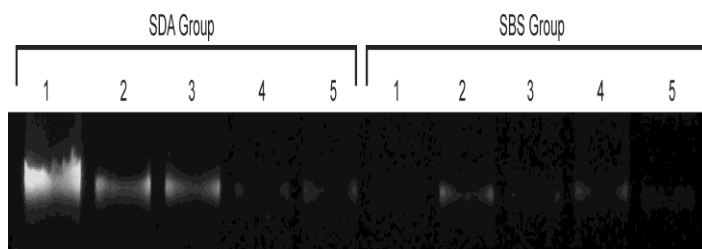


Figure 1. DNA quality evaluation by electrophoresis in 0.8% agarose gel. The SDA group was taken directly from the SDA samples; the SBS group was taken from stored SBS buffer samples.

C. albicans after storage in SDA and SBS were, respectively, 16.62 and 9.732 ng. Although these results are numerically different, the statistical analysis showed no significant differences between the two storage conditions. The qualitative evolution was based on 0.8% agarose gel observation (Figure 1), and showed no degradation pattern in the SDA and SBS storage conditions. It is important to highlight that the SDA and SBS groups were stored for two years prior to DNA extraction, demonstrating these storage conditions could represent good methods for maintaining the DNA of *C. albicans* for analysis.

Bacelo (2009) evaluated the phenotypical and molecular characteristics of *C. albicans* before and after 180 days of storage in SDA or distilled water. They carried out molecular evaluation using RAPD, a method routinely used to assess genetic relatedness of infectious fungi, which has been effectively applied to *C. albicans*. The fragment patterns obtained from SDA and distilled water samples were not significantly altered after storage, which corroborates in part the results obtained by our study.

The literature has pointed toward the use of SDA as a subculture method for *C. albicans* prior to DNA extraction, because the SDA culture media allows *C.*

albicans to grow and suppress the development of many species of oral bacterial. This method has shown promising results. Gurbuz and Kaleli (2010) isolated *C. albicans* from clinical specimens using SDA for a subculture preceding DNA extraction. From a total of 194 isolates obtained from 160 patients, they were able to genotype all strains, which demonstrates they had obtained high-quality DNA. However, although the standard protocol has been the use of *C. albicans* from SDA to extract DNA, the present result has shown the possibility of using samples directly from SBS to perform DNA extraction. This method could be more appealing, because it is less time consuming and has lower costs.

In conclusion, SDA and SBS methods could preserve *C. albicans* for DNA extraction to evaluate quantitative and qualitative data. However, determining if the DNA quality evaluated is adequate for molecular analysis will require more in-depth studies.

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Conflict of interest

The authors declare no potential conflicts of interest.

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