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Full Length Research Paper

In vitro antagonistic activity evaluation of some selected fungi isolated from burned soils in Mila region (East of Algeria)

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The present study was initiated to (i) determine burned forest-inhabiting fungi in Zouagha, TerriBeinène, Mila and (ii) study the antagonistic activities of *Trichoderma* sp against *Fusarium* sp, *Penicillium* sp, *Rhizoctonia* sp, and *Alternaria* sp. Eighteen fungal strains representing six genera were isolated from soil samples obtained from the burned forest of Zouagha in the Mila region: *Trichoderma* sp, *Fusarium* sp, *Penicillium* sp, *Rhizoctonia* sp, *Alternaria* sp, and *Rhizopus sp.* The direct antagonistic activity assays of *Trichoderma sp* on Potato Dextrose Agar medium (PDA) against the four fungi: *Fusarium* sp, *Penicillium* sp, *Rhizoctonia* sp, and *Alternaria* sp revealed that the fungus *Trichoderma* sp reduced the mycelium growth of *Fusarium* sp, *Penicillium* sp, *Rhizoctonia* sp and *Alternaria* sp to 23.13, 33.13, 33.75, and 38.31%, respectively, compared to the control after six days at room temperature. The results illustrated an inhibitory action of the antagonist *Trichoderma sp* characterized by slowing the mycelial growth of fungal strains. Strains of *Fusarium* sp, *Penicillium* sp, *Rhizoctonia* sp and *Alternaria* sp showed differences in the sensitivity to the antagonist. Because *Trichoderma* occurred more frequently in burned soils and were more antagonistic to phytopathogenic fungi in culture than isolates from unburned soils, the judicious use of fire may increase the abundance of *Trichoderma* isolates and their inhibitory action may be used for the control of fungal plant diseases.

Key words: Fungi, burned soil, Zouagha, Antagonism, *Trichoderma* sp.

INTRODUCTION

Forest ecosystems of cork and oak often present a balance of extreme complexity and their burning generate a cascade of degradations, which spread over many years and sometimes prove to be irreversible. Certainly, the trees which are weakened after a wildfire, present the ideal conditions for massive colonization by various species of fungi. Some of these are phytopathogenic fungi (Belhoucine and Bouhraoua, 2013). Some microorganisms are considered to be more sensitive to heat than others, for example fungi compared to bacteria

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Figure 1. The canton of BeniAfak (forest Zouagha, Mila) [source: Google Earth].

(Gema et al., 2011).The forest fire is one of the most widespread disruption and potentially most destructive affecting fungi that inhabit the soil (Lygis et al., 2010). The genus *Trichoderma* includes a set of saprophytic imperfect fungi which are commonly found in soil, dead wood, plant debris and aerial parts of the plants. They are easily recognized in the culture through the generally greenish color of their spores and their typical phialides (bowling-like).

The antagonistic properties of *Trichoderma* have long been recognized since the first publication in 1887. However, the comprehensive study of the phenomenon of antagonism and its application as a means to fight crop pests started between the two world wars (Johanne, 2002). *Trichoderma* has a set of potentially usable mechanisms for attacking, but remain complex. It can use one or more modes of action simultaneously to control a pathogen. The deployment of modes of action also varies according to the partners involved and the physical and chemical conditions of the medium (temperature, humidity, etc). *Trichoderma* is effective when it is allowed to install before the pathogenic fungi. Therefore its action is preventive (Johanne, 2002). In this study, the effects of selected *Trichoderma* sp isolated from burned soils on the growth and development of *Fusarium* sp, *Penicillium* sp, *Rhizoctonia* sp and *Alternaria* sp were evaluated.

MATERIALS AND METHODS

Study site and sample collection

The work focuses on the study of antagonistic fungi isolated from burned forests in Mila region. Soil, bark and leaves samples were collected from different sites in the forest Zouagha which is bound from the north by Djijal, the west by OuledRabeh, the south by BeniHaroune and the east by Grarem-Gouga. The study area is located on the topographical map of SidiMarouane scale 1/50.000, sheet N°50, between 804.8 to 805 altitude E and 366 à 366.2 longitude N. Z. Zouagha forest is spread over an area of 353, 50 ha and is entirely limited and divided into five regions: El Bahloul, Bouzourane, DjbelDaya, Arres and BeniAfek (Figure 1).

Sampling was conducted on February 19, 2014 at 11: 30 am. Three random sites of burned forest of Zouagha, BeniAfek region in Terri Beinène, Mila were selected. Samples were taken from tree bark and 1 kg of soil (after the removal of 3 cm of the upper layer of soil) of each site and as well as leaves from site 2 to evaluate the presence of fungi. After that, they were stored at 4°C in cooler, then transported to the laboratory until use.

Isolation and purification of fungi

Isolation of fungal strains was carried out according to the suspension dilution method (Davet and Rouxel, 1997). One gram of the soil sample, from each site, was aseptically added to 9 ml sterile saline water. The suspension was vortexed and diluted up to 10^{-6}. The bark and leaves samples were washed first in bleach for 5 min, then with ethanol for 5 min to remove microorganisms from the surface. Purification of strains was done on PDA agar (pH =5.1). Plates were incubated at 25°C for 6 days.

Identification of fungi

Strain's identification was conducted following the conventional dichotomous identification scheme. The macromorphology identification was done on the basis of colonies' properties of the isolation media. The micromorphology of the isolates were determined by direct light microscopic examination at 10X and 40X (optical microscope EXACTA+OBTEC) according to the determination keys of Botton et al. (1990).

Antagonism test

Direct confrontation method also called opposite cultures technique was used to determine antagonism activity. In a Petri dish containing 15 ml of PDA medium, two agar pellets (8 mm in diameter) of antagonist and pathogen were placed 4 cm from each other. Petri dishes containing pathogenic fungi were used as control. The plates were incubated at a temperature of 25°C and continuous light as an activation factor of certain enzymes. The

Table 1. Source of isolates and their frequencies.

Fungal isolate	Site	Leaf	Bark	Soil	Frequency %
*Trichoderma sp*1		-	-	+	
*Trichoderma sp*2		-	-	+	
Fusarium sp1	**1**	-	-	+	33.33
*Trichoderma sp*3		-	-	+	
*Trichoderma sp*4		-	-	+	
*Trichoderma sp*5		-	-	+	
*Trichoderma sp*6		-	-	+	
*Fusarium sp*2		-	-	+	
Alternaria sp	**2**	+	-	-	27.77
*Trichoderma sp*7		-	-	+	
*Rhizoctonia sp*1		-	-	+	
*Rhizoctonia sp*2		-	+	-	
Rhizopus sp		-	-	+	
*Trichoderma sp*8		-	-	+	
*Fusarium sp*3	**3**	-	-	+	38.88
Penicillium sp		-	-	+	
*Trichoderma sp*9		-	-	+	
*Trichoderma sp*10		-	-	+	

*(+) Presence of fungi, *(-) Absent of fungi.

development of mycelia growth was monitored every 24 h by measuring diameters of mycelial colony in millimeter. The percentage of mycelial growth inhibition was determined using the following formula (Hmouni et al., 1996):

$$I\ (\%) = (1 - Cn/Co) \times 100$$

I(%): is the percentage of mycelia growth inhibition
Cn: is the average diameter of the colonies in the presence of the antagonist
Co: average diameter of the control colonies.

RESULTS

Isolation and identification of fungi

Fungi were isolated from almost all analyzed samples and were identified. Eighteen fungal strains belonging to six genera: *Alternaria* sp, *Fusarium* sp, *Penicillium* sp, *Trichoderma* sp, *Rhizoctonia* sp and *Rhizopus* sp were isolated. Results are illustrated in the Table 1. The isolation results demonstrate that the highest frequency of the fungal isolate which is 38.88% is from site number 3. Five different genera including *Rhizoctonia* sp2, *Rhizopus* sp, *Trichoderma* sp 8, 9, 10, *Penicillium sp,* and *Fusarium* sp3 were isolated. The site number 1 had 33.33% , including *Trichoderma sp*1, 2, 3, 4, 5, and *Fusarium sp*1 and finally the site number 2 had 27.77%; *Trichoderma* sp6, 7, *Fusarium* sp2*, Alternaria* sp, and *Rhizoctonia* sp1.

The percentages of fungal isolates varied; 16.66% for *Fusarium* sp, 5.55% for *Alternaria sp,* 55.55% for *Trichoderma* sp, 5.55% for *Rhizopus* sp, 11.11% for *Rhizoctonia* sp and 5.55% for *Penicillium* sp (Tables 2 and 3).

Antagonism

The results of antagonism test of *Trichoderma* against four fungal isolates (*Alternaria* sp, *Fusarium* sp, *Penicillium* sp, and *Rhizoctonia* sp) show a medium reduction of mycelial growth of colonies of different fungal isolates compared to control (Figure 2 and Table 4). The tested colonies of *Trichoderma* sp inhibited the germination of *Fusarium* sp conidia by 23.13%, followed by *Penicillium* sp by 33.13%, *Rhizoctonia* sp *by* 33.75% and *Alternaria* sp by 38.31% (Figure 3).

DISCUSSION

In the study, the fungus flora of the burned forest soil of Zouagha, Mila region was determined. Among the genera obtained *Trichoderma* sp, *Fusarium* sp, *Penicillium* sp, *Rhizoctonia* sp, *Alternaria* sp, and *Rhizopus* sp.

Lucarotti (1981) obtained higher frequencies of *Trichoderma, Penicillium, Mucor*Mich ex Fr. and

Table 2. Samples analysis of unburned soil from Zouagha forest.

Parameter / Sample	Content expressed as % by weight of dry materials				
	Carbonates NF P(94-48)	Sulfates	MO	CO_2	pH
Site 01	40.08	Traces	15,56	1.70	6.08
Site 02	40.27	Traces	6.14	1.30	6.27
Site 03	41.41	Traces	5.30	0.80	6.41

Table 3. Samples analysis of soil obtained from Zouagha forest.

Parameter / Sample	Content expressed as % by weight of dry materials				
	Carbonates NF P(94-48)	Sulfates	MO	CO_2	pH
Site 01	4.88	Traces	10.0	2.15	7.0
Site 02	6.32	Traces	4.0	2.78	7.13
Site 03	6.32	Traces	4.0	2.78	7.80

Figure 2. Representative images of controls of five fungal genera after six days.

Table 4. The effect of *Trichoderma sp* on *Fusarium sp*, *Penicillium sp*, *Rhizoctonia sp* and *Alternaria sp.*

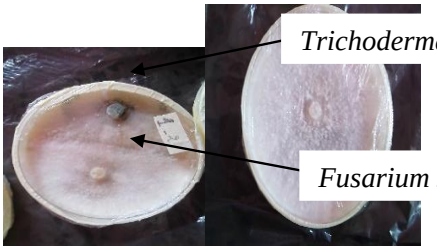
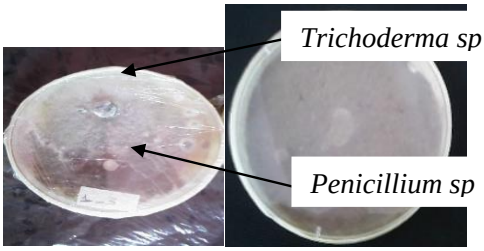
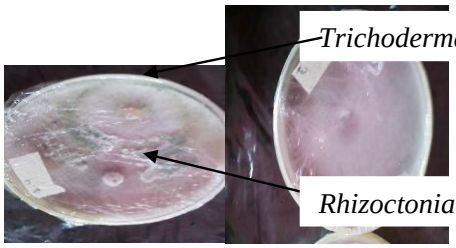
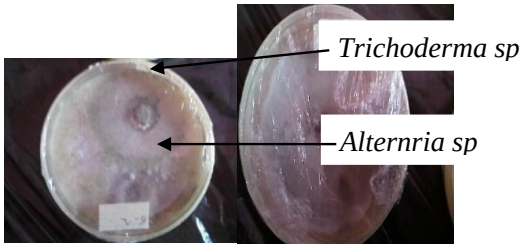
Antagonist – Pathogen	Antagonistic activity	
Trichoderma sp + Fusarium sp		T2
Trichoderma sp + Penicillium sp		T3

Table 4. Contd.

Trichoderma sp + Rhizoctonia sp		T4
Trichoderma sp + Alternria sp		T5

T2 :*Fusarium sp*, **T3 :***Penicillium sp*, **T4 :** *Rhizoctonia sp* and **T5:** *Alternaria sp.*

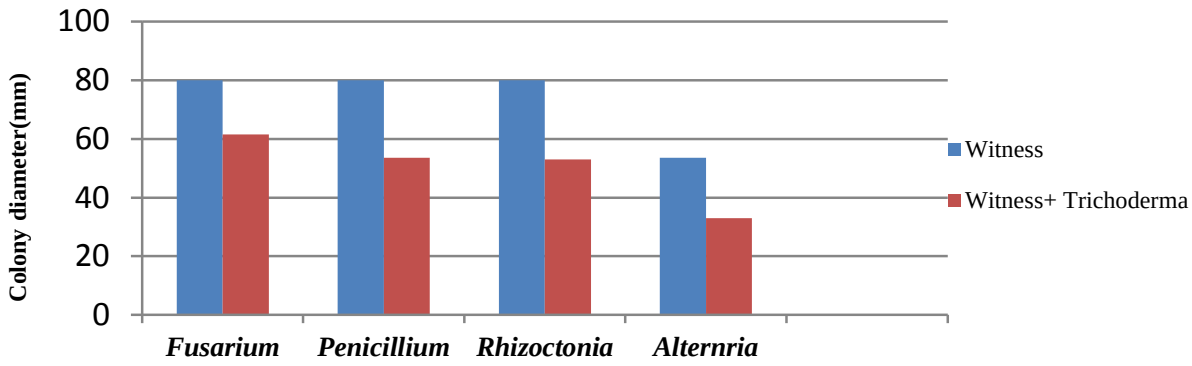


Figure 3. Colony diameter of *Fusarium sp*, *Penicillium sp*, *Rhizoctonia sp* and *Alternaria sp* in the presence of *Trichoderma sp* after 6 days.

Mortierella Coemans at in soil of burned forest in Canada. It can be postulated that these species do not show much sensitivity to ecological extremes and are more resistant to negative conditions. Also, Reaves et al. (1990) reported that they obtained *Trichoderma citrinoviride* Bissett most frequently in burned forest soil. Chwalinski (1989) determined that the variety of species following a fire was renewed within a year but the fungal density was not renewed completely in this period.

Many researchers reported that the soil humidity, soil pH (RamaRao, 1970), salt amount (Hasenekoglu and Sulun, 1990), and organic matter content (Behera and Mukerji, 1985) influence the activity of soil microorganisms. The fact that the amount of organic matter is very high in all soils proves that the rapidly spread fire, did not do much harm underground and the fire was only on the surface (Hasenekoglu and Sulun, 1990).

In addition, 20% of organic matter is nitrogen, and thus these soils are considered to be very rich in nitrogen. This may have a positive effect on microorganism activity in the soil (Table 2). The fact that the soil has a low rate of salt and lime (Ca^{+2}) could exclude their negative effect on the activity of soil microorganisms.

Suciatmih (2006) found that a significant positive correlation existed between the fungal population and the total organic carbon content. Waid (1960), listed temperature, humidity, CO_2, oxygen concentration, size of the soil pores, longevity of fungal mycelium, interaction between soil fauna and soil fungi and soil reaction as

factors that may influence growth and production of the mycelium of fungi in the soil.

According to Suciatmih (2006), the forest fire leads to a reduction and possibly an elimination of soil fungi. Chet (1984) reported studies on mode of action of *Trichoderma* used for biological control against *Rhizoctonia solani* in the case of cotton and strawberry cultivation, the results highlighted the importance of mycoparasitism phenomenon in the effectiveness of *Trichoderma*.

In the case of direct confrontation between *Alternria alternata* and *Trichoderma harzianum. A. alternria* has a very short development time but *T. harzianum* grows faster and surrounds the pathogen on the second day. *T. harzianum* develops without obstacles, and it has opportunities to stop the development of *A. alternate*. It grows over the colony of the pathogen at the same time (Biljana and Jugosslave, 2011; Gveroska and Ziberosk, 2011).

Rajendiran et al. (2010) demonstrated the inhibitory effect of *Trichoderma viride* against *Fusarium* sp., *Penicillium* sp. And *Aspergillus* sp. Growth inhibition of these fungi is due to its rapid growth nature, extracellular secretion of harmful compounds such as antibiotics, enzymes that can degrade cell wall such as gluconases, endochitinases, chitinases and mycoparasitism. Harman et al. (2004) described the mycoparasitic action of *Trichoderma* sp against pathogens. It coils around the hyphae of pathogen and produced peptaible which facilitate the entry of hyphae of *Trichoderma* sp into the lumen of parasitic mold.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Behera N, Mukerji KG (1985). Seasonal variation and distribution of microfungi in forest soils of Delhi India. Folia Geobot. Phytotx. 20:291-311.

Belhoucine L, Bouhraoua RT (2013). Les champignons phytopathogènes susceptibles de s'installer en subéraies post-incendie. Laboratoire De Recherche « Gestion Conservatoire De L'eau, Sol Et Forêts » -Tlemcen.2013.

Biljana G, Jugoslav Z (2011). *Trichodermaharzianum* as a biocontrol agent against *Alternariaalternata* on tobacco. Appl. Technol. Innov. 7:67-76.

Botton B, Breton A, Fevre M, Guy PH, Larpent JP, Reyond P, Sanglier JJ, Vayssier Y, Veau P (1990). Moisissures utiles et nuisibles importance industrielle.

Chet L (1984). Application of Trichoderma as a biocontrol agent. Proceeding. 6th Congress. Un. Phytopathology. Mediterrean, Cairo, Egypt pp.110-111.

Chwalinski K (1989). Effect of a soil surface fire on the soil mycoflora in a Scots pine forest. Prace-z-Zakresu-Nauk-Lesnych 64:17-23.

Davet P, Rouxel F (1997). Détection et isolement des champignonsdu sol. Ed INRA. Paris.

Gema BM, Fuensanta GO, Jorge MS, Jorge MB, Erland B (2011). Soil microbial recolonisation after a fire in a Mediterranean forest. Biol. Fertil. Soils 47:261-272.

Gveroska B, Ziberosk J (2011). *Trichodermaharzianum* as a biocontrol agent against *Alternariaalternata* on tobacco. Appl. Technol. Innov. 7:67-76.

Harman G, Howel CR, Viterbo UN, Chet J, Mlorito (2004). *Trichoderma* espèce-opportuniste, les avirulent plantent des symbiotes. La nature en examen 2:43-56.

Hasenekoglu I, Sulun Y (1990). Erzurum Askale cimento fabrikasinin kirlettigi topraklarin mikrofungus florasi uzerine bir arastirma. Turk. J. Bot. 15:20-27.

Hmouni A, Hajlaoui MR, Mlaiki A (1996). Résistance de *Botrytis cinerea*aux benzimidazoles et aux dicarboximides dans les cultures abritées de tomate en Tunisie. OEPP/EPPO Bull 26:697-705.

Johanne C (2002). Le pouvoir antagoniste de *Trichoderma*. Conférence présentée lors des journées horticoles régionales à St-Rémi.

Lucarotti C (1981). The effect of fire and forest regeneration on mesofauna population and microfungal species in lichens. McGill Subarctic Res. Stn. Publ. Meteorol. 32:7-26.

Lygis V , Vasiliauskaite I , Stenlid J , Vasaitis R (2010). Impact of forest fire on occurrence of *Heterobasidionannosum*s.s. root rot and other wood-inhabiting fungi in roots of *Pinusmugo.* Forestry 83:84-91.

Rajendiran R, Jegadeeshumar D, Sureshkumar BT, Nisha T (2010). In vitro assessment of antagonistic activity of *Trichodermaviride* against post-harvest pathogens. J. Agric. Technol. 6:31-35.

RamaRao P (1970). Studies on soil fungi III. Seasonal variation and distribution of microfungi in some soils of Andhra pradesh (India). Mycopathol. Mycol. Appl. 40:277-298.

Reaves JL, Shaw CG, Mayfeild JE (1990). The effect of *Trichoderma spp*. isolated from burned and non-burned forest soils on the growth and development of *Armillariaostoyae* in culture. Northwest Sci. 6:39-44.

Suciatmih S (2006). Soil Fungi in an Over-burned Tropical Rain Forest in Bukit Bangkirai, East Kalimantan. Biodiversitas 7:1-3.

Waid JS (1960). The growth of fungi in soil. In: Parkinson D. and Waid J.S (ed.). The Ecology of Soil Fungi. Leverpool: Liverpool University Press.

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Full Length Research Paper

Inhibition of biofilms on urinary catheters using immobilized *Lactobacillus* cells

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Bacterial biofilms forming on indwelling urinary catheters continue to represent a public health problem because they are associated with urinary tract infections (UTIs). This study was undertaken to evaluate the ability of immobilized *Lactobacillus acidophilus* cells to inhibit biofilm formation on catheter surfaces. Urine bacteria and *Lactobacillus* species were isolated from urine and vaginal swabs (HVS), respectively. Immobilization of *L. acidophilus* on catheter samples was achieved using sodium alginate and the inhibition of urine bacteria by the immobilized *Lactobacillus* cells was evaluated by microscopy and viable cell count procedures following co-culture of the immobilized cells and urine bacteria. Results showed that pre-coating of catheter surfaces with *L. acidophilus* before exposure to urine bacteria significantly ($p<0.05$) reduced attachment of some urine bacteria to the catheter surfaces. *Staphylococcus aureus, Klebsiella* and *Escherichia coli* were significantly inhibited, while *Pseudomonas aeruginosa* was not inhibited. Furthermore, crude bacteriocin preparations from the *Lactobacillus* cells had antimicrobial activity against the urine bacteria. This study shows that pre-coating of catheter surfaces with *L. acidophilus* could be an effective strategy for controlling biofilm formation on urinary catheters.

Key words: Biofilm, catheter, *Lactobacillus*, immobilization, sodium alginate.

INTRODUCTION

Urinary tract infections (UTIs) account for about 25-40% of nosocomial infections, out of which about 90% are catheter-associated (Trautner and Darouiche, 2004; Ghanwate et al., 2012). The daily incidence of bacteriuria in catheterized patients is approximately 3–10% and among patients with bacteriuria, up to 25% will develop symptoms of local UTI, while about 3% will develop bacteremia (Saint et al., 2005). Studies have also shown that the duration of catheterization is the important factor of bacteriuria (Stickler, 2008; Al-Mathkhury et al., 2011).

Several strategies have been attempted to control urinary catheter biofilms including application of antimicrobial ointments and lubricants, bladder instillation or irrigation, antimicrobial agents in the collection bags, impregnation of catheter with antimicrobial agent such as silver oxide or use of systemic antibiotics, but none of these strategies proved very effective (Donlan, 2001; Trautner and Darouiche, 2004). Currently, novel

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strategies are being attempted by researchers, including coating of catheters with enzymes, EDTA, biosurfactants, probiotics and other non-pathogenic organisms (Darouiche et al., 2001; Trautner et al., 2007; Borchert et al., 2008; Fracchia et al., 2010; Ghanwate et al., 2012; Sambanthamoorthy et al., 2014).

Lactobacilli are probiotic bacteria known to have a positive effect on the maintenance of human health and are considered to be generally safe (Merk et al., 2005; Borchert et al., 2008). These bacteria, which constitute an important part of the natural microbiota, are recognized as potential interfering bacteria. In particular, lactobacilli have long been known for their antimicrobial activity and capability to interfere with adhesion of pathogens to epithelial cells of the urogenital tract (Reid et al., 2001; Reid and Burton, 2002; Borchert et al., 2008; Fracchia et al., 2010; Ali 2012; Sambanthamoorthy et al., 2014), hence their application in vaginal suppositories for the treatment of vaginal and urinary tract infections (Reid and Burton, 2002; Uehara et al., 2006; Borchert et al., 2008). This property of bacterial interference of lactobacilli therefore presents a potential intervention strategy for the control of urinary catheter biofilms. Some investigators have reported attempts to inhibit biofilm-formation by applying *Lactobacillus*-derived bio-surfactants, acid supernatants, bacteriocin or whole cells to surfaces colonized by biofilm-forming bacteria (Maldonado et al., 2007; Fracchia et al., 2010; Al-Mathkhury et al., 2011; Sambanthamoorthy et al., 2014). In pre-coating and co-incubation assays, these researchers showed that more effective inhibition was achieved with higher concentrations of the *Lactobacillus*-derived substances.

Alginates are hydrocolloids and water-soluble biopolymers, which have been applied for immobilization and proliferation of cells (Andersen et al., 2012). This study evaluated the capability of *Lactobacillus acidophilus* cells, immobilized on catheter surfaces with sodium alginate, to inhibit the formation of biofilms by *Escherichia coli, Klebsiella, Staphylococcus aureus* and *Pseudomonas aeruginosa in vitro.*

MATERIALS AND METHODS

Collection of samples

Sterile Foley silicone catheters were purchased from the market; urine specimens were collected aseptically from outpatients attending a local hospital and from apparently healthy individuals for the isolation of urine bacteria; and high vaginal swab (HVS) specimens were collected from an apparently healthy female for isolation of *Lactobacillus* spp.

Isolation and identification of organisms

In order to isolate urine bacteria, loopfuls of the urine samples were cultured on MacConkey and Blood agar and incubated for 24 h at 37°C. Resultant colonies were sub-cultured onto fresh culture plates of the same media to obtain pure cultures. Then, isolates were characterized and identified, using standard microbiological procedures as described by Cheesebrough et al. (2000).

For *Lactobacillus* spp., the vaginal swab specimens were inoculated onto De Man Rogosa Sharpe (MRS) agar (Oxoid). The plates were incubated at 37°C in an anaerobic jar, for 24 to 48 h. Thereafter, the creamy-white colonies, suspected to be *Lactobacillus,* were sub-cultured on MRS medium to obtain pure cultures. The isolates were further characterized and identified by biochemical analyses as described by Cheesebrough et al. (2000).

Evaluation of growth of *Lactobacillus* in urine

Ten milliliters of urine was filter-sterilized to make the urine free from bacteria. The sterile urine was then used as a culture medium for *Lactobacillus* cells using an inoculum size of 10^6 cfu. The culture was incubated at 37°C in an anaerobic jar. Viable cell counts were taken from triplicate samples, each day, for seven days, to evaluate the survival and growth of the cells in urine.

Evaluation of anti-biofilm effect of *Lactobacillus* using cover-slip assay

The ability of *Lactobacillus* cells to inhibit biofilm formation by urine bacteria was evaluated qualitatively by cover-slip assay by a modification of the method of Ghanwate et al. (2012). Sterile Petri dishes were filled each with 10 ml of MRS broth containing 1% sucrose. Then, sterile glass cover-slips were added to each Petri dish. Thereafter, each plate was inoculated with 0.1 ml of overnight culture of *L. acidophilus* and incubated anaerobically at 37°C for 24 h to coat the cover-slips with *Lactobacillus*. The next day, the *Lactobacillus*-coated cover-slips were extracted and introduced into culture dishes containing 10 ml broth-culture of different urine isolates (*S. aureus, E. coli, Klebsiella* sp. and *P. aeruginosa*) to produce co-cultures of the *Lactobacillus* and urine organisms. The dishes were then incubated aerobically at 37°C. On daily basis, a cover-slip from each culture set-up was removed, unattached cells were rinsed off with phosphate buffered saline and attached cells or biofilms were stained with 0.1% crystal violet for 5 min (for *S. aureus*) and Gram staining for *E. coli*, *Klebsiella* sp. and *P. areuginosa*. Finally, stained biofilms were observed microscopically and photographed using a 0.2 Mega pixel Motic camera. The co-cultures were monitored over a seven-day period.

Immobilization of *Lactobacillus* acidophilus on catheter pieces

MRS broth containing *L. acidophilus* cells was incubated for 48 h at 37°C. The broth was then centrifuged at 5000 rpm for 15 min. The supernatant was discarded while the pellet of cells was added into a beaker containing exactly 2% (w/v) sodium alginate solution. The catheter pieces were then introduced into the mixture and allowed to stand for 1 h. Thereafter, the catheter pieces were extracted and immersed in a beaker containing 2% $CaCl_2.2H_2O$ to allow the formation of a gel. The set-up was incubated for 24 h for stability of the gel.

Evaluation of anti-biofilm capability of immobilized *Lactobacillus* cells

The catheter pieces containing immobilized *L. acidophilus* cells were immersed in broth cultures of the different urine isolates in separate Bijoux bottles. The bottles were allowed to stand for 7 days at 37°C. Untreated catheter sections also immersed in broth cultures of urine isolates served as control. Biofilm formation on the catheter sections was evaluated by viable cell count procedures.

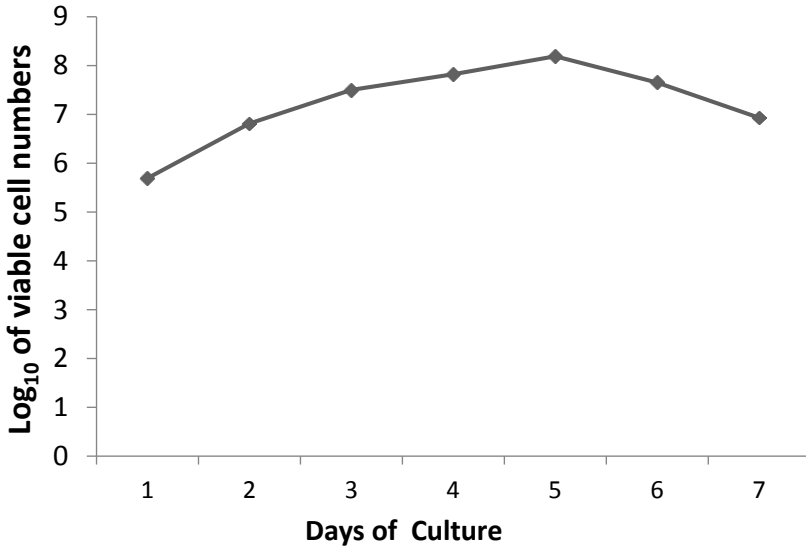

Figure 1. Growth of *L. acidophilus* in urine over a period of 7 days.

On each day of counting, two catheter pieces were picked from the various cultures and rinsed with sterile distilled water to remove unattached cells. Then, the attached cells were gently scraped off from both the outer and luminal surfaces of the catheters using a wire loop and introduced into sterile beakers containing 10 ml of phosphate buffered saline. The cells in the biofilm were dispersed using a magnetic stirrer. Serial dilutions were prepared and the cells inoculated onto MacConkey and blood agar plates for viable counts.

Preparation of crude bacteriocin of *L. acidophilus*

The isolated *L. acidophilus* was propagated in a 100 ml-conical flask containing 25 ml MRS broth and incubated anaerobically at 37°C for 24 h. Cells were separated by centrifugation at 5000 rpm for 20 min at room temperature. The supernatant was adjusted to pH 7 by using 1 N NaOH to remove the antimicrobial effect of organic acids. The clear solution was then filtered using 0.22 µ pore size filter.

Determination of antimicrobial activity of crude bacteriocin

The inhibitory activity of the crude bacteriocin preparation against the urine isolates was determined using disc diffusion method. Paper discs of 6 mm diameter were prepared from Whatman No.1 filter paper. The discs were autoclaved for 15 min at 121°C and allowed to cool. Then, the sterile discs were impregnated with the bacteriocin preparation. The impregnated discs were placed on solidified Muller-Hinton agar seeded with 18 h cultures of the test organisms and incubated at 37°C for 24 h. The assay was performed in triplicates. Zones of inhibition were then measured and the values were recorded.

Statistical analysis

Analysis of variance (ANOVA) and $LSD_{0.05}$ were employed to analyze the data on viable counts using Microsoft Excel 2007 application.

RESULTS

Isolation of bacteria from urine and HVS

Four bacteria were isolated from the urine specimens. These were: *S. aureus, E. coli, Klebsiella* sp. and *P. aeruginosa.* All four organisms were used in the biofilm studies. Two *Lactobacillus* species were identified from HVS, namely: *L. acidophilus* and *Lactobacillus plantarum.* The *L. acidophilus* was used for all the anti-biofilm studies.

Growth of *Lactobacillus* in urine

L. acidophilus cells were able to survive and multiply in urine, increasing in number from 6.5×10^7 to 1.5×10^8 cfu/ml by the fifth day of culture, after which there was a decrease in number (Figure 1).

Inhibition of urine bacteria by *Lactobacillus* cells on coverslips

Evaluation of the inhibitory activity of *Lactobacillus* cells against bacterial biofilms on coverslips showed that pre-coating of coverslip surfaces with *Lactobacillus* cells prior to exposure of the surfaces to urine bacteria reduced the number of urine bacterial cells attaching to the coverslips. For *E. coli*, cell numbers reduced relative to number of *Lactobacillus* cells from the third day of culture, while there was complete inhibition by the sixth day (Figure 2). Similarly, numbers of attached cells of *Klebsiella* and *S. aureus* reduced from the third day and complete inhibition was achieved by the seventh day (Figures 3 and 4). For

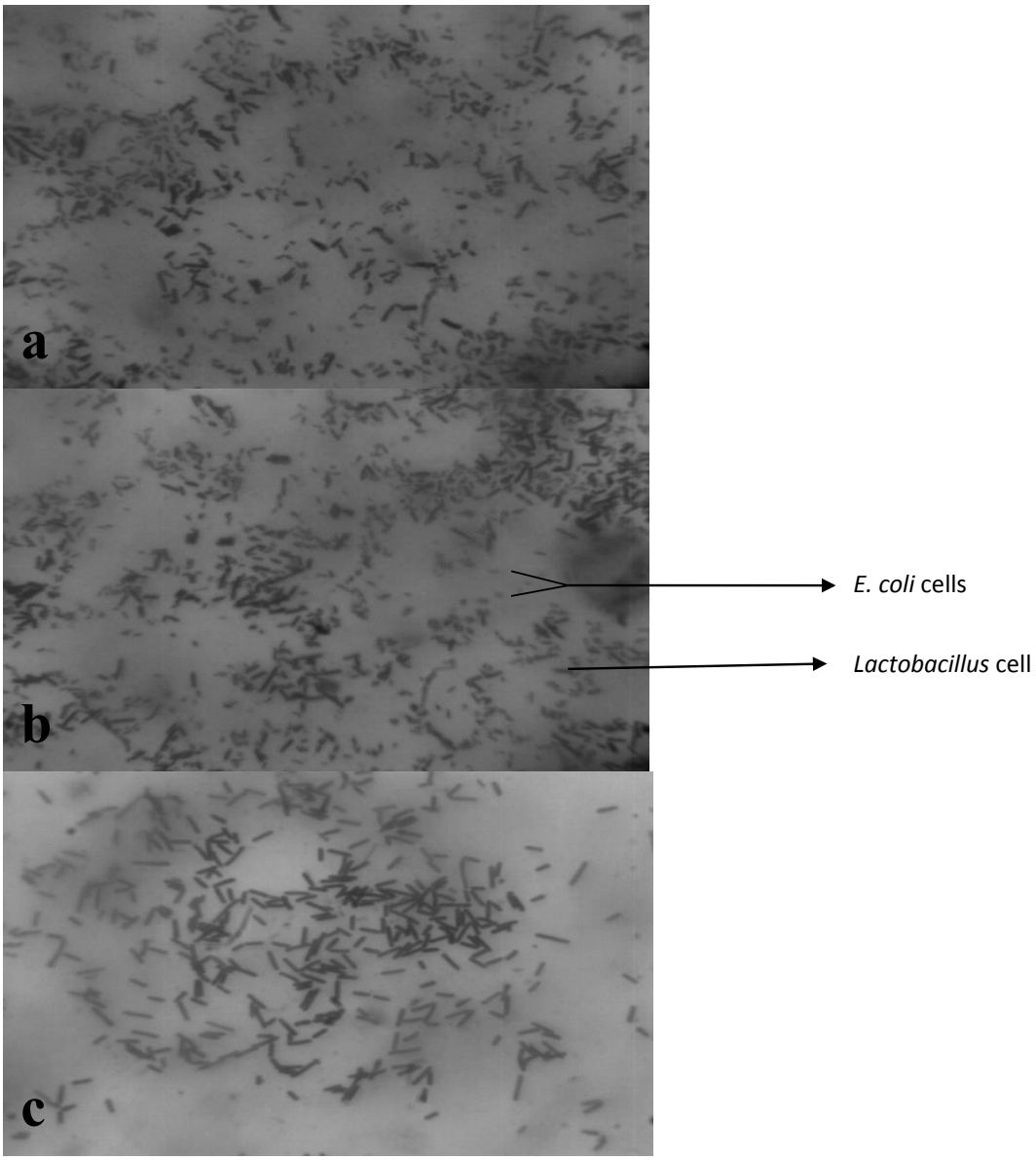

Figure 2. Microscopic images of co-culture of *L. acidophilus* and *E. coli* on coverslip; a, Day 1; b, Day 3; c, Day 6. Note the presence of a mixture of short rods of *E. coli* and large rods of *Lactobacillus* in panels a and b, and the presence of predominantly large rods of *Lactobacillus* in panel c.

P. aeruginosa, on the other hand, reduction in number was observed only from the fifth day and attached cells were still observed by the end of the experiment on the seventh day (Figure 5).

Inhibition of catheter biofilms by immobilized *Lactobacillus* cells

Evaluation of attachment and growth of urine organisms on catheter pieces containing immobilized *Lactobacillus* cells was compared with growth on uncoated catheter pieces. The results showed significant ($p<0.05$) reduction in number of *S. aureus* and *Klebsiella* cells on the coated catheter pieces when compared with the uncoated catheters. *E. coli* and *P. aeruginosa*, on the other hand, showed no reduction. Representative graphs of the growth of *S. aureus* and *P. aeruginosa* are shown in Figures 6 and 7, respectively.

Inhibitory activity of crude bacteriocin of *L. acidophilus* against test isolates

The crude bacteriocin preparation from the *Lactobacillus* isolate was tested for its activity against the urine

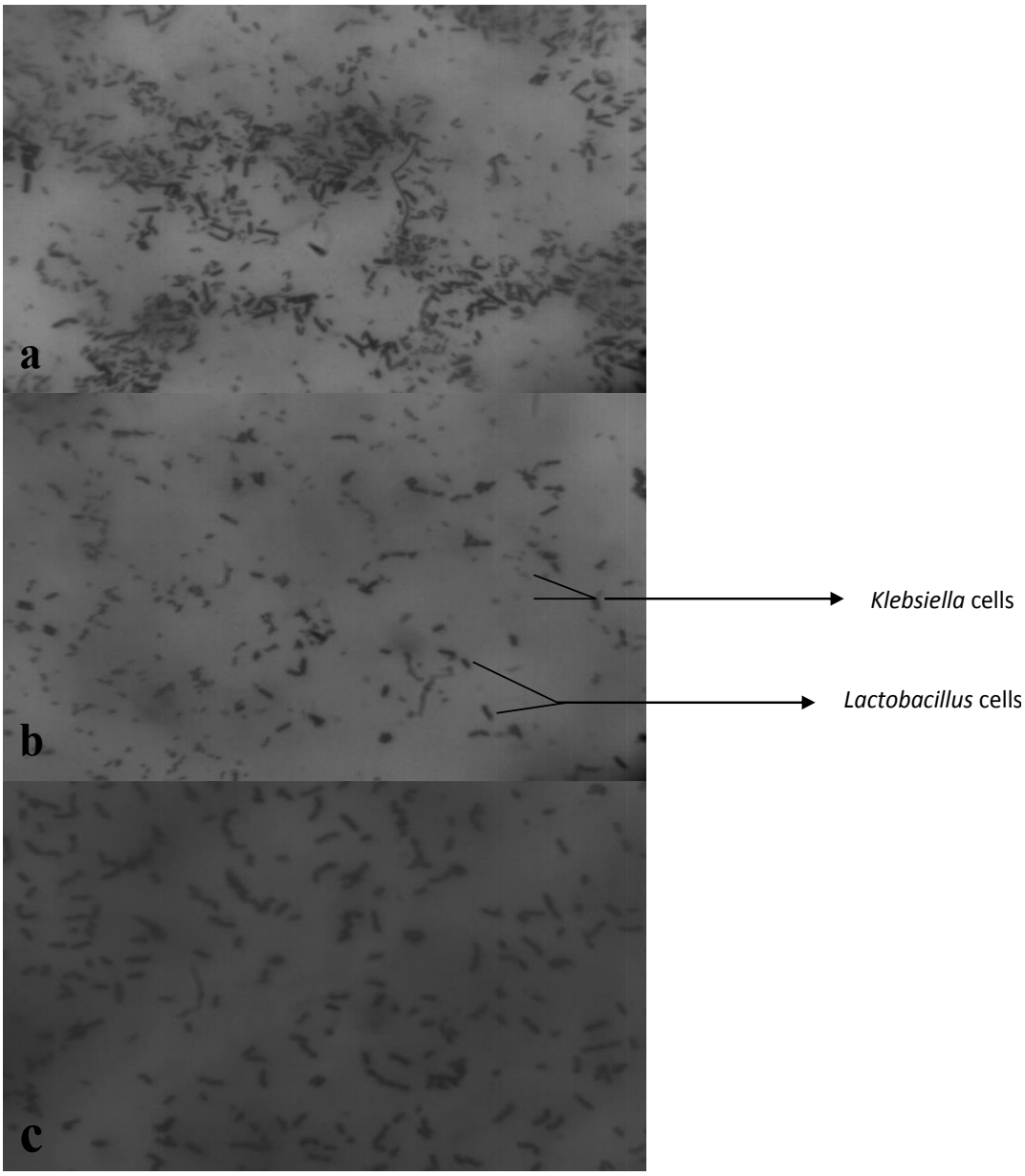

Figure 3. Microscopic images of co-culture of *L. acidophilus* and *Klebsiella* on coverslip; a, Day 1; b, Day 3; c, Day 6. Note the presence of a mixture of short rods of *Klebsiella* and large rods of *Lactobacillus* in panels a and b, and the presence of predominantly large rods of *Lactobacillus* in panel c.

isolates. The results showed that the highest activity was against *S. aureus,* followed by *Klebsiella* before *E. coli* (Figure 8).

DISCUSSION

The human normal bacterial flora is increasingly recognized as an important defense to infection. Lactobacilli in particular, are regarded to be generally safe and recognized as potent interfering bacteria. Lactobacilli and their derived substances have been used widely in both clinical and experimental trials to inhibit growth of other bacteria on various natural and artificial surfaces, including urinary catheters (Reid and Tieszer, 1994; Reid and Burton, 2002; Reid and Bruce, 2006; Maldonando et al., 2007; Ruiz et al., 2009; Barrons and Tassone, 2008; Fracchia et al., 2010; Ray, 2011; Al-Mathkhury et al., 2011; Sambanthamoorthy et al., 2014; Abd-Alkareem, 2014).

In this study, *L. acidophilus* was isolated from HVS of a healthy female. The *Lactobacillus* cells showed appreciable growth in urine for up to 5 days in culture, after which there was a decline in growth. The decline in growth after five days could be attributed to culture

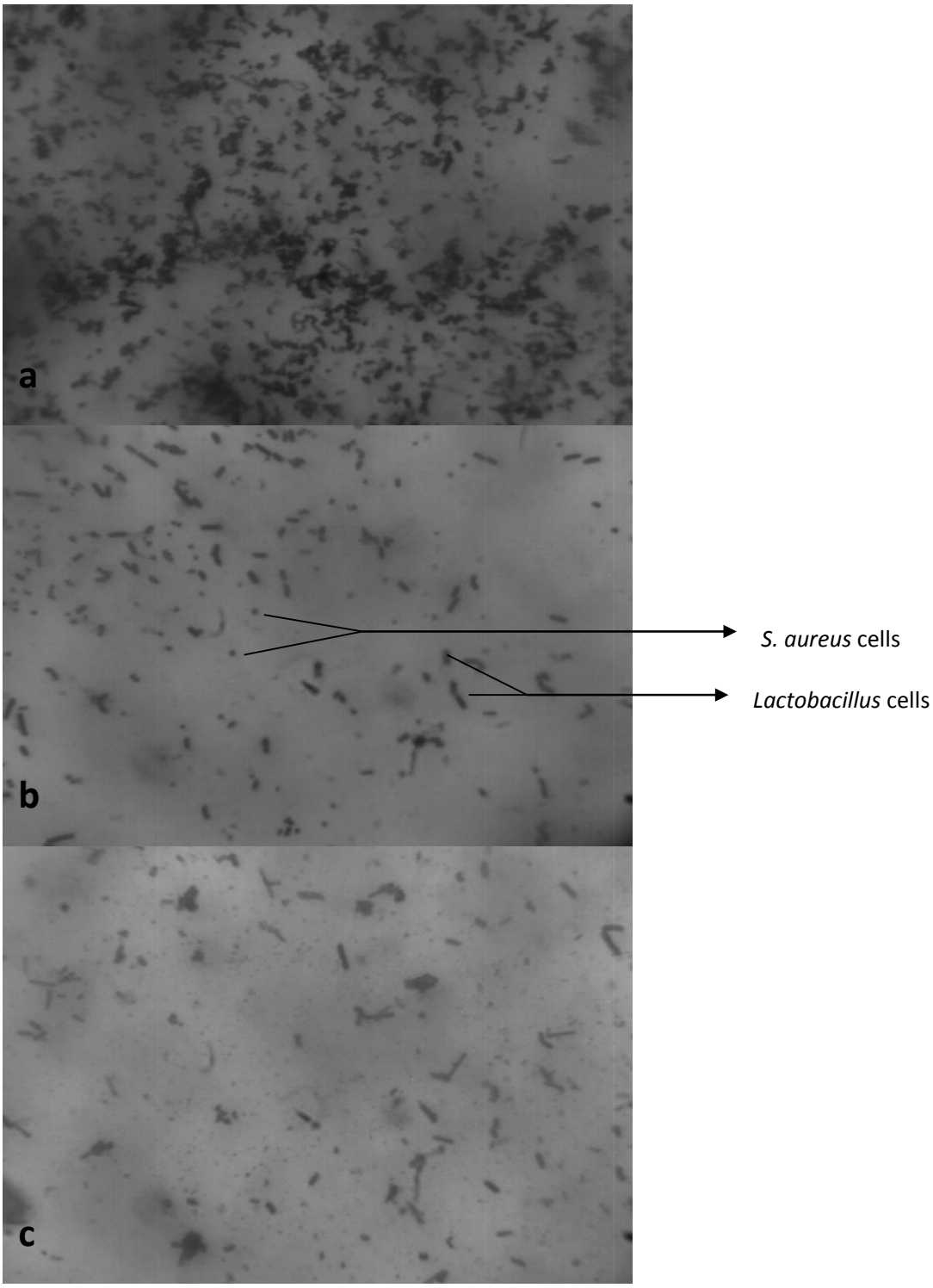

Figure 4. Microscopic images of co-culture of *L. acidophilus* and *S. aureus* on coverslip; a, Day 1; b, Day 3; c, Day 7. Note the presence of a mixture of spherical cells of *S. aureus* and large rods of *Lactobacillus* in panels a, b and c.

conditions, in which the cells had to survive in what could be described as stagnant urine.

Evaluation of the anti-biofilm potential of the *Lactobacillus* cells by cover-slip assay showed that pre-coating of the surfaces with *Lactobacillus* reduced the attachment and growth of urine bacteria. The effect was

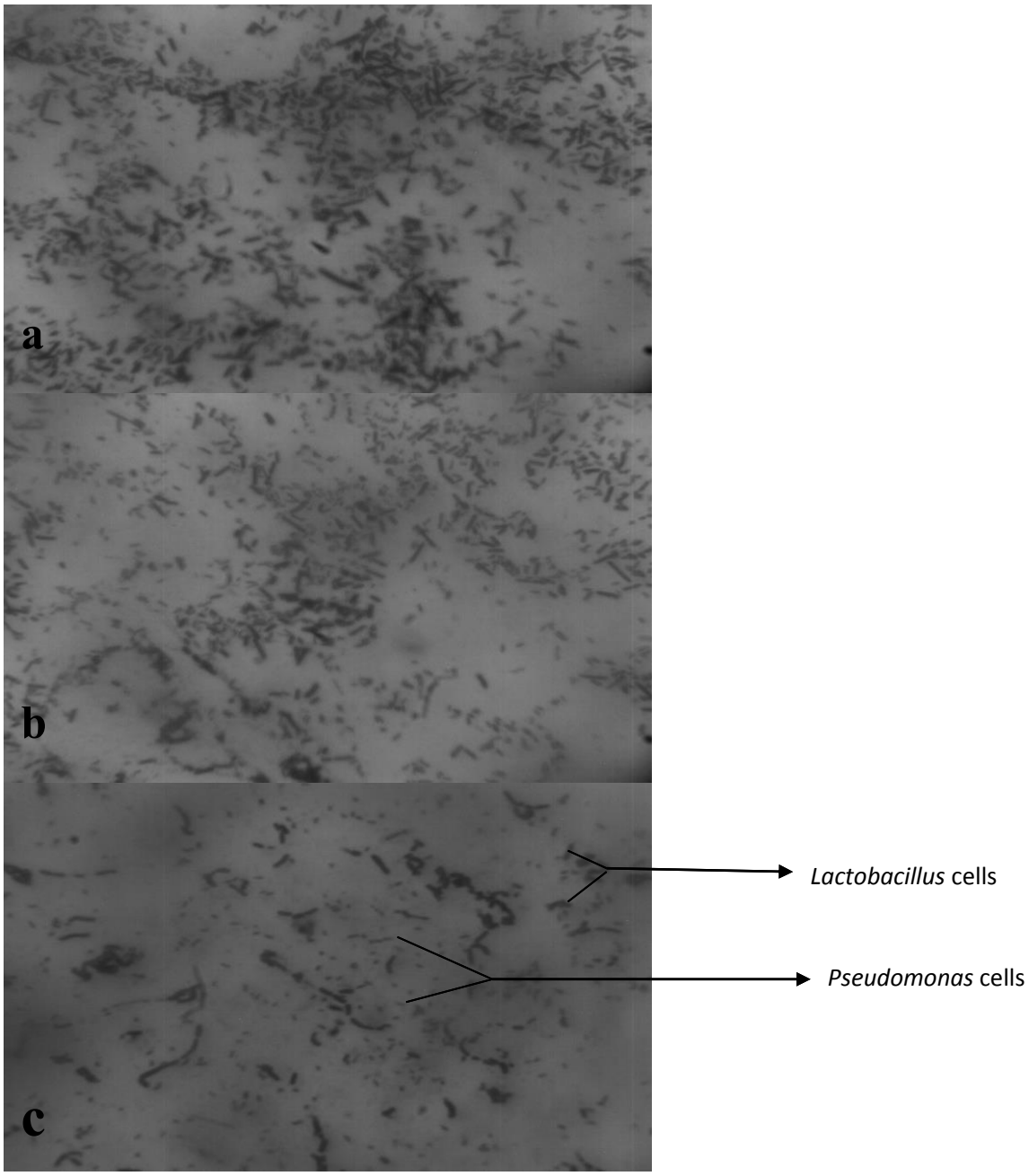

Figure 5. Microscopic images of co-culture of *L. acidophilus* and *P. aeruginosa* on coverslip; a, Day 1; b, Day 3; c, Day 7. Note the presence of a mixture of slim rods of *Pseudomonas* and large rods of *Lactobacillus* in panels a, b and c.

more pronounced against *E. coli*, *Klebsiella* and *Staphylococcus*, with near complete inhibition being achieved by the fifth day of co-culture. For *P. aeruginosa*, on the other hand, clearing required longer than seven days. The inhibitory effect observed in the cover-slip assay was confirmed quantitatively using viable cell count procedures following immobilization of *Lactobacillus* cells on catheter surfaces and exposure to urine bacteria. In the quantitative assay, however, more inhibition was recorded with *S. aureus* and *Klebsiella* than with *E. coli* and *Pseudomonas*.

The results in this study agree with those of Maldonado et al. (2007), in which whole cells and acid supernatant of *Lactobacillus fermentum* inhibited biofilm formation and growth of *Klebsiella.* Westbroek et al. (2010) reported that *Streptococcus pyogenes* was not inhibited by *Lactobacillus crispatus* and *Lactobacillus jensenii.* In Westbroek's (2010) study, the *Lactobacillus* and

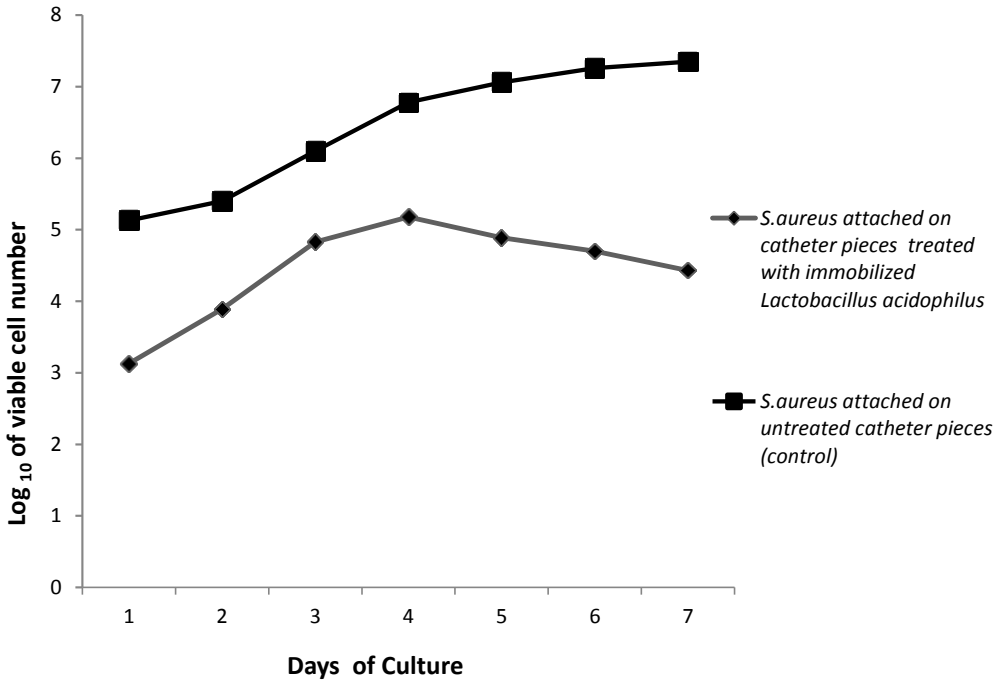

Figure 6. Growth of *S. aureus* on untreated catheter and catheter material pre-coated with *L. acidophilus.*

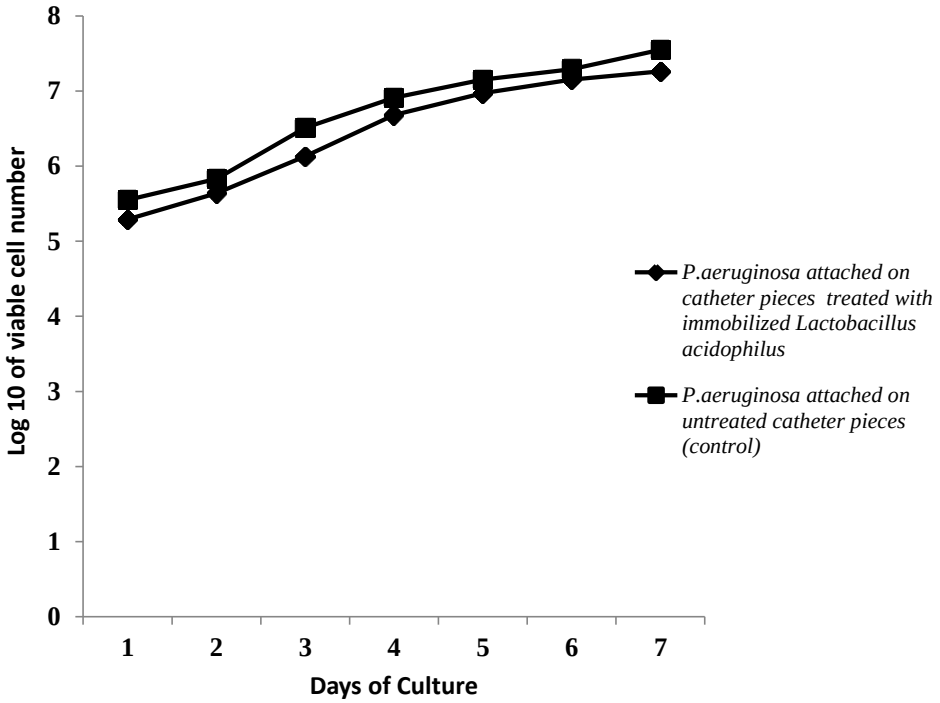

Figure 7. Growth of *P. aeruginosa* on untreated catheter and catheter material pre-coated with *L. acidophilus.*

Steptococcus cells were mixed before plating. It appears therefore that the inhibitory action requires pre-application of the *Lactobacillus* or its products to the surface before exposure to the biofilm-forming organism, as was

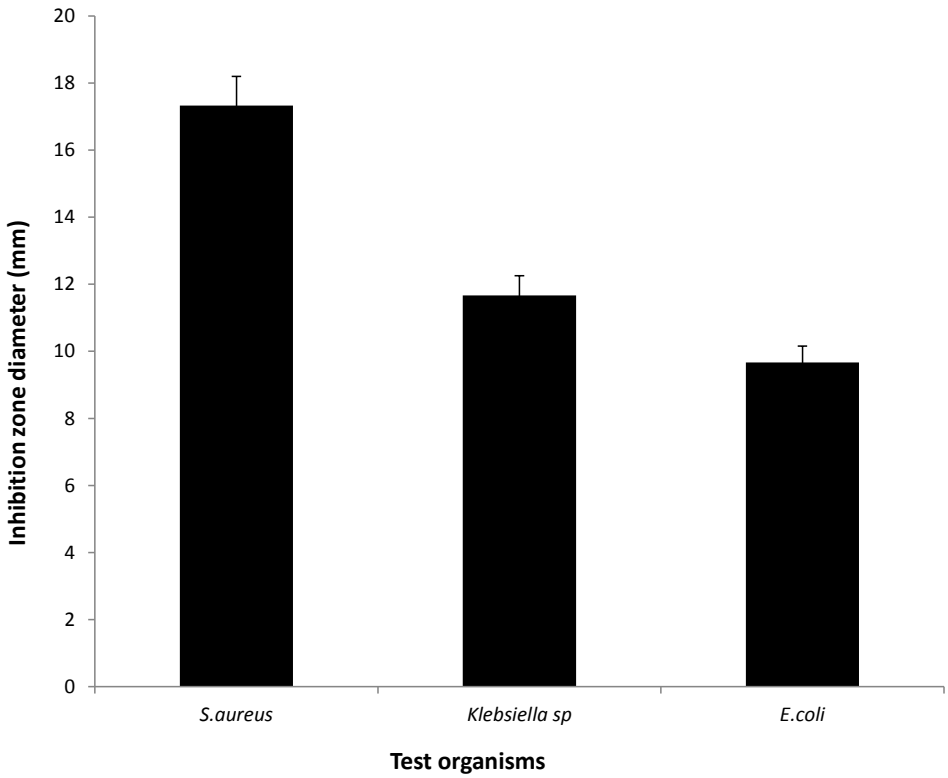

Figure 8. Inhibitory activity of crude bacteriocin from *L. acidophilus* against some test bacteria.

demonstrated in the study by Fracchia et al. (2010). Similar results were also reported by Hall et al. (2000) and Darouiche et al. (2001), working with benign *E. coli* strains. In their studies, instillation of the benign *E. coli* strains into the bladders of patients with recurrent UTI and coating of urinary catheters with benign *E. coli,* respectively, significantly reduced the occurrence of UTI in the patients.

In the present study, cell-free supernatant from the *L. acidophilus* was also evaluated for its activity against the urine bacteria by disk-diffusion method and the results showed that *S. aureus, Klebsiella* and *E. coli* were inhibited, while *Pseudomonas* was resistant.

The results from this preliminary study showed that *Lactobacillus* has the potential to inhibit urinary catheter biofilms when applied to catheters and the inhibitory action could be both by removal of attachment surface for the urine bacteria and production of antibacterial substances.

The finding presents an interesting intervention strategy that should be explored further for actual use. Concerns that may come to mind in consideration of practical application are those relating to safety and stability of alginate, blockage of the catheter by the coating, as well as possibility of infection by the *Lactobacillus.* These questions would be answered by carrying out further trials using small animals.

Conflict of interest

There is no conflict of interest.

REFERENCES

Abd-Alkareem AY (2014). *Lactobacillus acidophilus* as antibiofilm formed by *Staphylococcus aureus in vitro.* Diyala. J. Med. 7:24-34.

Ali OA (2012) Inhibition of uropathogenic *Citrobacter* adhesion by biosurfactants extracted from vaginal *Lactobacillus acidophilus.* J. Al-anbar Univers. Med. Sci. 10:11-19.

Al-Mathkhury HJF, Ali AS, Ghafil JA (2011). Antagonistic effect of bacteriocin against urinary catheter associated *Pseudomonas aeruginosa* biofilm. N. Am. J. Med. Sci. 3:367-370.

Andersen T, Strand BL, Formo K, Alsberg E, Chritensen BE (2012). Alginates as biomaterials in tissue engineering. Carbohydr. Chem. 37:227-258.

Barrons R, Tassone D (2008). Use of *Lactobacillus* probiotics for bacterial genitourinary infections in women: a review. Clin. Therapeutics 30:453-468.

Borchert D, Sheridan L, Papatsoris A, Faruquz Z, Barua JM, Junaid I, Pati Y, Chinegwundoh F, Buchholz N. (2008). Prevention and treatment of urinary tract infection with probiotics: Review and research perspective. Indian J. Urol. 24:139-144.

Cheesebrough M (2000). District Laboratory Practice in Tropical Countries. Tropical Health Technology and Butterworth-Heinemann Ltd. Cambridge. 2:63-70.

Darouiche RO, Donovan WH, Del Terzo M, Thornby JI, Rudy DC, Hull RA (2001). Pilot trial of bacterial interference for preventing urinary tract infection. Urology 58:339-344.

Darouiche RO, ThornbyJI, Cerra-Stewart C, Donovan WH, Hull RA (2005). Bacterial interference for prevention of urinary tract

infection:A prospective, randomized,placebo-controlled, double blind pilot trial. Clin. Infect. Dis. 41:1531-1534.
Donlan RM (2001). Biofilms and device-associated infections. Emerg. Infect. Dis. 7:277-280.
Fracchia L, Cavallo M, Allegrone G, Martinotti MG (2010). A *Lactobacillus*-derived biosurfactant inhibits biofilm formation of human pathogenic *Candida albicans* biofilm producers. Curr. Res. Technol. Educ. Top. Appl. Microbiol. Microb. Biotechnol. 2:827-837.
Ghanwate NA, Thakare PV, Bhise PR, Dhanke A, Apotikar S (2012). Prevention of biofilm formation in urinary catheter by coating enzymes/gentamycin/EDTA. World Acad. Sci. Eng. Technol. 6:468-470.
Hall R, Rudy D, Donovan W, Svanborg C, Wieser I, Stewart C, Darouiche R (2000). Urinary tract infection prophylaxis using *Escherichia coli* 83972 in spinal cord injured patients. J. Urol. 163:872-877.
Maldonado NC, Silva de Ruiz C, Cecilia M, Nader-Macias ME (2007). A simple technique to detect *Klebsiella* biofilm-forming strains. Inhibitory potential of *Lactobacillus fermentum* CRL 1058 whole cells and products. Commun. Curr. Res. Educ. Top. Trends Appl. Microbiol. pp. 52-58.
Merk K, Borelli C, Korting HC (2005). *Lactobacillus*-bacteria-host interactions with special regards to the urinary tract. Int. J. Med. Microbiol. 295:9-18.
Ray K (2011). Infection: *Lactobacillus* probiotic could prevent recurrent UTI. Nature Rev. Urol. 8:292.
Reid G, Tieszer C (1994). Use of lactobacilli to reduce the adhesion of *Staphylococcus aureus* to catheters. Int. Biodeterior. Biodegrad. 34:73-83.
Reid G, Bruce AW (2002). Probiotics to prevent urinary tract infections: the rationale and evidence. World J. Urol. 24:28-32.
Reid G, Bruce AW, Fraser N, Heinemann C, Owen J, Henning B (2001). Oral probiotics can resolve urogenital infections. FEMS Immunol. Med. Microbiol. 30:49-52.
Reid G, Burton J (2002). Use of *Lactobacillus* to prevent infection by pathogenic bacteria. Microb. Infect. 4:319-324.
Ruiz FO, Gerbaldo G, Asurmendi P, Pascual LM, Giordano W, Barberis IL (2009). Antimicrobial activity, inhibition of urogenital pathogens and synergistic interactions between *Lactobacillus* strains. Curr. Microbiol. 59:497-501.
Saint S, Kaufman SR, Thompson M, Rogers MA, Chenowith CE (2005). A reminder reduces urinary catheterization in hospitalized patients. Joint Commission J. Qual. Patient Safety 31:455-462.
Sambanthamoorthy K, Feng X, patel R, Patel S, Paranavitana C (2014). Antimicrobial and antibiofilm potential of biosurfactants isolated from lactobacilli against multi-drug-resistant pathogens. BMC Microbiol. 14:197.
Stickler DJ (2008). Bacterial biofilms in patients with indwelling urinary catheters. Nature Clin. Practice Urol. 5:598-608.
Trautner BW, Darouiche RO (2004). Role of biofilm in catheter-associated urinary tract infection. Am. J. Infect. Control 32:177-183.
Trautner BW, Hull RA, THornby JI, Darouiche RO (2007). Coating urinary catheters with an avirulent strain of *Escherichia coli* as a means to establish asymptomatic colonization. Infect. Control Hosp. Epidemiol. 28:92-94.
Uehara S, Monden K, Nomoto K, Seno Y, Kariyama R, Kumon H (2006). A pilot study evaluating the safety and effectiveness of *Lactobacillus* vaginal suppositories in patients with recurrent urinary tract infection. Int. J. Antimicrob. Agents 28:S30-34.
Westbroek ML, Davis CL, Fawson LS (2010). Interactions of lactobacilli with pathogenic *Streptococcus pyogenes.* Infect. Dis. Obstet. Gynecol. pp. 289-743.

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Full Length Research Paper

Characterization and purification of bacteriocin produced by *Enterococcus* sp. GHB26 isolated from Algerian paste of dates "Ghars"

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Strain *Enterococcus* sp. GHB26, isolated from the Algerian paste of dates "Ghars", produced a bacteriocin. This bacteriocin was inactivated by proteolytic enzymes. Antibacterial activity of the bacteriocin was heat stable at 120°C for 20 min (533 AU/ml), stable at pH range of 2 to 12 and resistant to chemicals (SDS, EDTA, NaCl, Tween 80, Urea). The active bacteriocin from the cell-free supernatant of *Enterococcus* sp. GHB26 was purified by precipitation with ammonium sulfate followed by various combinations of gel filtration on a Sephadex G-25 column, cation exchange chromatography on a CM-Sephadex Cellulose column and reverse phase-high performance liquid chromatography on a C18 column. The bacteriocin was eluted as a single peak on the chromatogram from reverse phase-high performance liquid chromatography attesting the purity of this bacteriocin. The bacteriocin exhibited a bactericidal mode of action. Sodium dodecyl sulphate - polyacrylamide gel electrophoresis indicated that molecular weight of this bacteriocin is close to 3.5 kDa.

Key words: Paste of dates, bacteriocin, antibacterial activity, chromatography, mode of action.

INTRODUCTION

Lactic acid bacteria had largely been exploited in the food fermentation and they display numerous antimicrobial activities (De Vuyst and Leroy, 2007; Šušković et al., 2010). Several lactic acid bacteria, mostly enterococci strains, are also found in the gastrointestinal tracts of humans and animals and are considered to exert health-promoting effects which include antimicrobial activity against pathogen strains. This is mainly due to the production of organic acids, diacetyl, hydrogen peroxide and acetaldehyde but also of other compounds, such as bacteriocins (Gürakan, 2007).

Bacteriocins are ribosomally synthesized peptides produced by bacteria that exhibit bactericidal or bacteriostatic effect against closely related bacteria but

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also against pathogenic bacteria (Collins et al., 2009). The bacteriocins are often cationic and amphiphilic or hydrophobic (Altuntas, 2013). The enterococcal bacteriocins are small heat stable proteins that kill closely related bacteria (Altuntas, 2013).

Bacteriocins from lactic acid bacteria have a wide potential applications such as, for example food biopreservatives and health care (Cotter et al., 2005; Šušković et al., 2010). Antimicrobial activity is often proposed as an important functional characteristic of probiotic strains, because bacteriocin biosynthesis by probiotics can contribute to eliminate undesirable bacteria during host colonization, thereby helping to prevent host from pathogen proliferation. Nisin and pediocin PA-1 produced by lactic acid bacteria are the only bacteriocins used in food preservation (Altuntas, 2013).

Before any use of a bacteriocin in food industry, its study must be done on biochemical and genetic characteristics, its antibacterial spectrum, its effectiveness in different food systems, and the regulatory implications (Javed, 2009). Each bacteriocin has unique properties and a particular efficiency in targeting microbial pathogens. This is why isolation and purification of new bacteriocins will be always beneficial (Elegado et al., 1997; Merzoug et al., 2015).The purification of bacteriocin uses different strategies depending on the properties of the producer bacteria and the experimental conditions available (Cintas, 1997), but it follows a common protocol that includes salt precipitation and various chromatography combinations like gel filtration, ion-exchange, hydrophobic-interaction, and reverse phase - high performance liquid chromatography from culture supernatants (Altuntas, 2013).

The aim of the present study was to characterize and investigate the antimicrobial activity of the cell-free supernatant of *Enterococcus* sp. GHB26 isolated from traditional date paste "Ghars" in different phases of bacteriocin purification. Furthermore, the interest was to find out the mode of action of the bacteriocin.

MATERIALS AND METHODS

Bacterial strains and media

In the present study, two strains from the Collection of the Laboratory of Biology of Microorganisms and Biotechnology, University of Oran, Algeria, were used: *Enterococcus* sp. GHB26 had been isolated from date paste "Ghars" (Dellali, 2012) and was used as producer of the bacteriocin and the strain *Enterococcus faecium* H3 (Lazreg et al., 2015), used as an indicator strain, had been isolated from "hammoum", a traditionally fermented barley. Strains were conserved by storage at -20°C in reconstituted skim milk (10% w/v). The strains were cultured in MRS broth medium (Fluka, Switzerland) at 30°C before use.

Detection of inhibitory activity

Bacteriocin screening was tested by the agar well diffusion method as described by Tagg and Mc Given (1971). 500 µL of a culture of the indicator strain *E. faecium* H3 (30°C, 18 h) was inoculated in 30 ml of MRS with 0.75% (w/v) agar and then poured onto plates. Aliquots of 60 µL from the cell-free supernatant, obtained after centrifuging a culture of the inhibitory strain *Enterococcus* sp. GHB26 (30°C, 18 h), were added to each well of the indicator lawn poured on MRS soft agar. After a diffusion step at 4°C overnight, the plates were incubated at 30°C for 24 h. The presence of the inhibition zone was considered as a positive result for bacteriocin production.

In order to exclude the inhibitory effect of lactic acid, the inhibitory strain *Enterococcus* sp. GHB26 was cultivated in MRS broth buffered with sodium phosphate buffer (0.1 M, pH 7). The activity of cell-free supernatant was defined as the reciprocal of the highest dilution value showing inhibition of the indicator strain and was expressed in arbitrary units per mililiter (AU/ml) (Graciela et al., 1995).

Physico-chemical characterization of the inhibitory substance

Preparation of cell-free supernatant

Enterococcus sp. GHB26 was cultivated 4% (v/v) in MRS broth buffered with sodium phosphate buffer (0.1 M, pH 7) and was grown for 18 h at 30°C. The cell-free supernatant was separated from cells by centrifugation at 3000 g for 15 min.

Effect of enzymes

Aliquots of 200 µL of the cell-free supernatant were incubated for 2 h at 37°C in the presence of 1 mg/ml of catalase, trypsin, pepsin, pronase E (Aktypis and Kalantzopoulos, 2003). All enzymatic solutions (Sigma-Aldrich) were prepared in buffer sodium phosphate (0.01 M, pH 7) except for pepsin which was dissolved in HCl of 0.02 M. Samples were boiled at 100°C for 5 min to stop the enzymatic reactions. Untreated cell-free supernatant was used as a control. The susceptibility of antibacterial activity to enzymes was evaluated by the agar well diffusion method (Tagg and McGiven, 1971).

Effect of temperatures

To determine the effect of temperature on the antibacterial activity, aliquots (200 µL) of cell-free supernatant were heated at 60, 80 and 100°C for 60 and 120 min and at 120°C for 20 min. The residual activity was evaluated according to Graciela et al. (1995) against *E. faecium* H3. The activity was expressed as arbitrary units (AU) per milliliter.

Effect of different pH

Aliquots of 200 µL of the cell-free supernatant were adjusted to different pH from 2 to 12 with HCl (5 M) or NaOH (5 M). After 4 h of incubation at 30°C (Hernandez et al., 2005), the residual antibacterial activity was tested as described before.

Effect of chemical agents

The chemical agents: SDS, Triton X-100, EDTA, NaCl, Tween 80 and Urea, were added separately (1% w/v) to the cell-free supernatant. After homogenization, the samples were incubated at 37°C for 5 h. Untreated cell-free supernatant was used as a control.

The residual antibacterial activity was tested by the agar well

diffusion method.

Purification of the bacteriocin GHB26

Precipitation by ammonium sulfate

Cell-free supernatant (140 ml) from *Enterococcus* sp. GHB26 was treated with ammonium sulfate to 40, 60 and 80% saturation. Mixtures were stirred at 4°C for 4 h and further centrifuged for 30 min at 3000 g. The precipitates were dissolved separately in 7 ml sodium phosphate buffer (0.02 M, pH 7) and investigated for their antimicrobial activity.

Sephadex G-25 Gel filtration chromatography

This chromatography separates molecules according to their size and it can be used for desalting. 1.2 ml of the precipitate obtained at 60% saturation of ammonium sulfate was applied to a Sephadex G-25 gel filtration column (26 cm length, 1.4 cm diameter, 5000 Da exclusion limit) that had been previously equilibrated with 0.1 M sodium phosphate buffer (pH 7). Elution was conducted using 0.1 M phosphate buffer at pH 7 at a flow rate of 0.5 mL/min. Fractions of 1 mL were collected and the absorbance measured at 280 nm. The inhibitory activity was determined by the agar well diffusion method. The active fractions were mixed and lyophilized.

Cation exchange chromatography on a CM- Sephadex Cellulose column

This column separates the peptides according to their electric charge at definite pH. The lyophilisate obtained from the gel filtration chromatography was suspended in 500 µL sodium phosphate buffer (0.1 M, pH 7) and separated by cation exchange chromatography through CM-Sephadex Cellulose column (10 cm length, 1.2 cm diameter) that had been previously equilibrated with 0.05 M sodium phosphate buffer (pH 7). Elution was conducted by a continuous gradient-elution (0-0.1 M NaCl, 0.1-1 M NaCl in 0.05 M phosphate buffer pH 7.0), at a flow rate of 0.5 ml/min. Fractions of 1 mL were collected and the absorbance measured at 280 nm. The inhibitory activity was determined and the active fractions were mixed and lyophilized.

Purification by reverse phase-HPLC

The lyophilisate obtained from the cation exchange chromatography was re-suspended in 250 µL of 0.1 M sodium phosphate buffer at pH 7 containing 10% (v/v) acetonitrile and purified by reverse phase-high performance liquid chromatography (RP-HPLC) on a column Inertsil ODS2 C18 (250 mm length, 4.6 mm internal diameter, 5 µm particle size). Elution was performed using a gradient of acetonitrile from 0 to 100% in 0.1% aqueous trifluoroacetic acid (TFA) for 30 min, at a flow rate of 1 mL/min. Eluted peaks were detected at 226 nm and the corresponding fractions were collected. The active fractions were mixed and lyophilized, then they were recovered and re-suspended in 50 µL phosphate buffer (0.02 M, pH7), and tested against the indicator strain *E. faecium* H3.

Estimation of the bacteriocin molecular weight by Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Schägger (2006), using 4% stacking gel, 10% spacer gel and 16% separating gel. The samples tested were the native supernatant, the concentrated supernatant, the fractions obtained after purification thanks to Sephadex G25, CM Sephadex Cellulose and HPLC. Molecular weight protein standards (Nisin 3.5 kDa and BSA 67 kDa) were used. SDS-PAGE was conducted overnight at 50V, 20 mA. After electrophoresis, the gel was cut vertically into two parts, the part of the gel that contained samples and protein markers was stained with a solution of Coomassie Brilliant Blue R-250 (Merck, Saint Louis, MO), while the remaining part containing only the samples was fixed in a mixture of 20% isopropanol (v/v), 10% acetic acid (v/v) for 1 h, and washed with sterile distilled water overnight and used for detection of antibacterial activity by overlaying with MRS soft agar inoculated with 800 µL of a 18 h culture of the indicator strain *E. faecium* H3 and incubated for 24 h at 30°C.

Mode of action of bacteriocin GHB26

The bacteriocin was tested for a bacteriostatic or bactericidal mode of action against *E. faecium* H3 as follows: 2 ml of concentrated bacteriocin was added to 10 ml growing cells of *E. faecium* H3 in MRS broth medium in early exponential phase (4 h at 30°C). A sample culture of *E. faecium* H3 without bacteriocin was used as a control. Optical density at 600 nm was determined at appropriate intervals.

RESULTS AND DISCUSSION

Detection of inhibitory activity

The strain *Enterococcus* sp. GHB26 inhibited the growth of indicator strain *E. faecium* H3. This antibacterial activity was recorded on buffered MRS broth with a maximum activity level of 1066 AU/ml, which excludes the possible effect of lactic acid. *Enterococcus* sp. GHB26 was found to produce an inhibitory substance against *E. faecium* H3.

Physico-chemical characterization of the inhibitory substance

Effect of enzymes

The antibacterial activity of the cell-free supernatant was sensitive to proteolytic enzymes, except pepsin, which indicated the proteinaceous nature of the active substance. The inhibitory activity was not affected by catalase, indicating that inhibition was not due to hydrogen peroxide production (Table 1). The inhibitory substance produced by *Enterococcus* sp. GHB26 may be considered as a bacteriocin, that we designated bacteriocin GHB26. Klaenhammer (1988) reported that the bacteriocins were sensitive at least for one proteolytic enzyme.

Effect of temperature and pH

The antibacterial activity of the inhibitory agent was

Table 1. Effect of enzyme, temperature, pH and chemical agents on inhibitory activity.

Treatment	Inhibitory activity (AU/ml)
Untreated	1066
Enzymes	
Catalase	1066
Trypsin	0
Pronase E	0
Pepsin	1066
Temperature	
60°C at 60 min	1066
60°C at120 min	1066
80°C at 60 min	1066
80°C at120 min	1066
100°C at 60 min	1066
100°C at120 min	1066
120°C at 20 min	533
pH	
2	1066
4	1066
6	1066
8	1066
10	1066
12	533
Chemical agents	
SDS (1%)	1066
Tween 80 (1%)	1066
Urea (1%)	1066
NaCl (1%)	1066
EDTA (1%)	1066
TritonX-100(1%)	0

stable to heat treatment at 60, 80 and 100°C for 60 and 120 min (1066 AU/ml). However, heating at 120°C reduced the activity to 50% after 20 min (533 AU/ml) (Table 1).This was in line with enterococcal bacteriocins that were reported to be heat stable (Park et al., 2003). The antibacterial activity was maintained stable over the range of pH from 2 to 10 (1066 AU/ml). However, a 50% loss of this activity at pH 12 (533 AU/ml) was noticed (Table 1). Similar results were observed for the bacteriocins produced by *E. faecium* ST5Ha (Todorov et al., 2010) and *E. faecium* GHB21 (Merzoug et al., 2015). The resistance to heat and to a wide pH range is an important characteristic for the application of this bacteriocin GHB26 as food biopreservative.

Effect of chemical agents

Treatment of cell-free supernatant with different chemical agents such as SDS, Urea, NaCl, Tween 80, EDTA did not affect the antibacterial activity of the bacteriocin GHB26, except Triton X100 (Table 1). Von Mollendorff et al. (2006) noticed a total loss of antibacterial activity of the bacteriocin JW11BZ from *Lactobacillus fermentum* after treatment with Triton X-100.

Purification of bacteriocin GHB26

Precipitation by ammonium sulfate

Maximum antibacterial activity was obtained when the cell-free supernatant was precipitated with 60% ammonium sulfate saturation (2132 AU/ml). Similar result was observed by Ogunbanwo et al. (2003) for the bacteriocin produced by *Lactobacillus plantarum* F1.

Sephadex G-25 gel filtration chromatography

Several fractions were collected (Figure 1). Only three

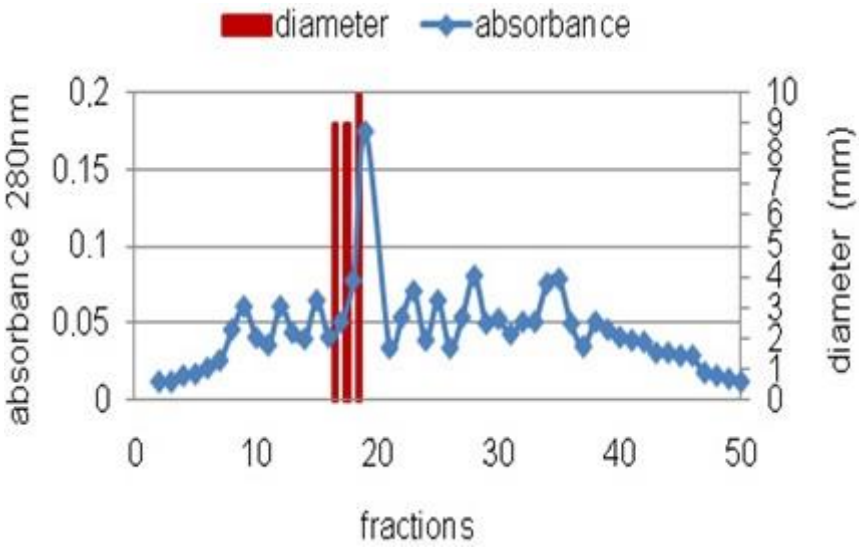

Figure 1. Sephadex G25 chromatogram of the active fraction from precipitation with ammonium sulfate at 60%.

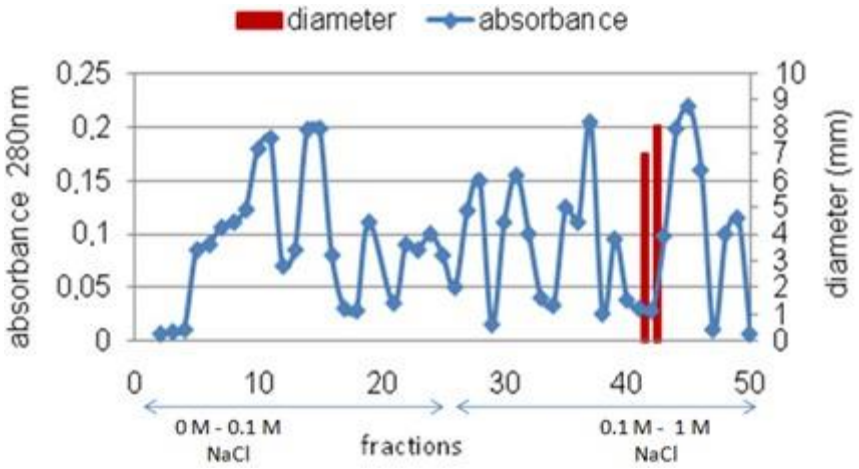

Figure 2. CM-Sephadex cellulose chromatogram of concentrated active fractions from Sephadex G-25 column.

fractions (16-17-18) have shown inhibitory activity against *E. faecium* H3. Sephadex G-25 column can separate proteins ranging in molecular weight from 1000 to 5000 Da. Obtained results suggest that the inhibitory substance has a molecular weight lower than 5000 Da since it was eluted with the elution volume. Similar result was reported by Smaoui et al. (2009) for the bacteriocin BacTN635 produced by *L. plantarum* TN635.

Cation exchange chromatography in a CM- Sephadex Cellulose column

Several fractions were collected (Figure 2). Only two fractions (42-43) have shown inhibitory activity against *E. faecium* H3. The active substance obtained from cation exchange chromatography was not eluted with a NaCl concentration of 0 to 0.1 M, but at a gradient of 0.1 to 1 M. This result shows that the bacteriocin GHB26 is cationic. Vera Pingitore et al. (2007) reported that in a standard protocol, the bacteriocin extract is passed through the cation exchange column; the bacteriocin eluted using a gradient of NaCl from 0.1 to 1 M.

Purification by reverse phase-HPLC (RP-HPLC)

RP-HPLC chromatogram showed a single peak (Figure 3).

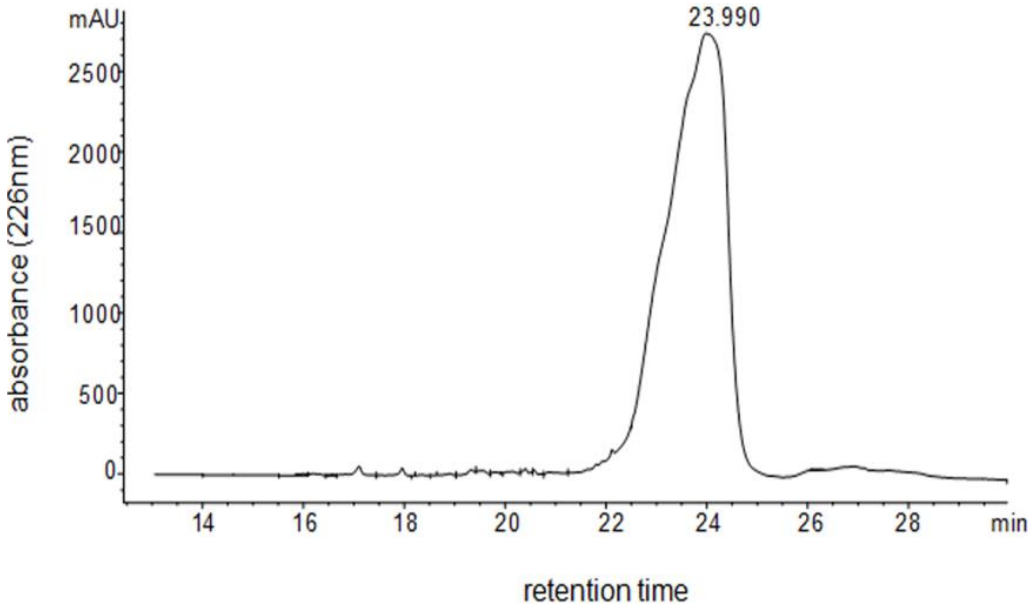

Figure 3. RP-HPLC Chromatogram of concentrated active fractions from CM- Sephadex Cellulose column.

This peak was eluted at 85% of acetonitrile, corresponding to a retention time of 23.99 min. The peak was shown to be active against *E. faecium* H3. This final purification step by RP-HPLC indicates the purity of the bacteriocin GHB26. Earlier reports (Elegado et al., 1997; Srinivasan et al., 2012) have obtained a single peak by RP-HPLC and these authors explain such a result by a high purity of the pediocin AcM from *Pediococcus acidilactici* M and the bacteriocin produced by *Lactobacillus rhamnosus* L34. However, it was observed that the bacteriocin GHB26 was eluted at 85% acetonitrile, which would correspond to an elution of hydrophobic protein (Kawai et al., 1994).

Using nisin as a standard, its elution was recorded to a retention time of 26 min. Verdon (2009) showed that the bacteriocins that have the same size and the same hydrophobicity properties will elute by RP-HPLC at very near time; this is the case of the bacteriocin GHB26 (23.99 min) and nisin (26 min).

SDS-PAGE for molecular weight determination

A Coomassie blue-stained SDS-PAGE gel revealed a diffuse band of the concentrated supernatant (Figure 4a). A comparison of the band on the stained gel with the half gel showing inhibition zones (Figure 4b) indicates that the band contained the active bacteriocin GHB26. In the case of native supernatant, no protein band was detected despite the presence of the inhibition zone. This zone was very close to nisin zone, corresponding to about 3.5 kDa. For the other samples (fractions obtained after purification by Sephadex G25, CM Sephadex cellulose and HPLC) no bands were observed on the stained gel and no zone of inhibition was observed on the native gel. BSA is observed in lanes 1 and 2 of the gel.

SDS-PAGE electrophoresis showed that the bacteriocin GHB26 has a molecular weight close to that of nisin (3.5 kDa).This result confirms what was previously observed by RP-HPLC. The obtained molecular weight of bacteriocin GHB26 averages within the size range of most bacteriocins from enterococcal strains. For example, enterocin 1071B produced by *E. faecalis* BFE 1071 was reported to have a molecular weight of 3.899 kDa (Balla et al., 2000), enterocin MC13 from *E. faecium* MC13 was reported to be 2.148 kDa (Satish Kumar et al., 2011).

Mode of action of bacteriocin GHB26

The optical density of the indicator strain culture remained constant after the addition of the bacteriocin (Figure 5). Hence the mode of action of the bacteriocin GHB26 was considered as bactericidal. Similar results were reported for bacteriocins produced by *E. faecium* LR/ 6 (Kumar et al., 2009) and *Lactobacillus salivarius* SMXD51 (Messaoudi et al., 2012).

Conclusion

Enterococcus sp GHB26 was able to produce an effective bacteriocin with interesting features. The

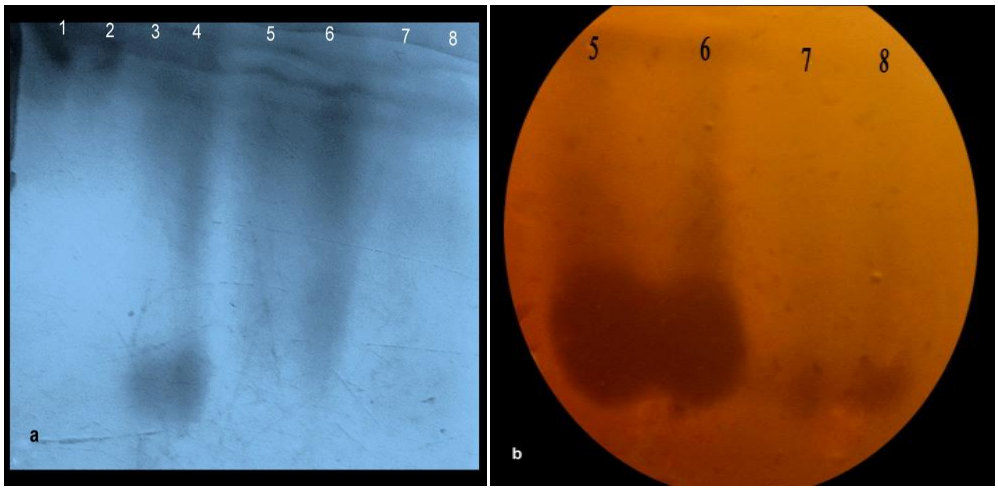

Figure 4. a. SDS-PAGE gel stained with Coomassie blue; b. Bacteriocin activity in native gel. Lane 1: BSA (30 µl), lane 2: BSA (60 µl), lane 3: nisin (30 µl), lane 4: nisin (60 µl), lane 5: concentrated supernatant (30 µl), lane 6: concentrated supernatant (60 µl), lane 7: native supernatant (30 µl), lane 8: native supernatant (60 µl).

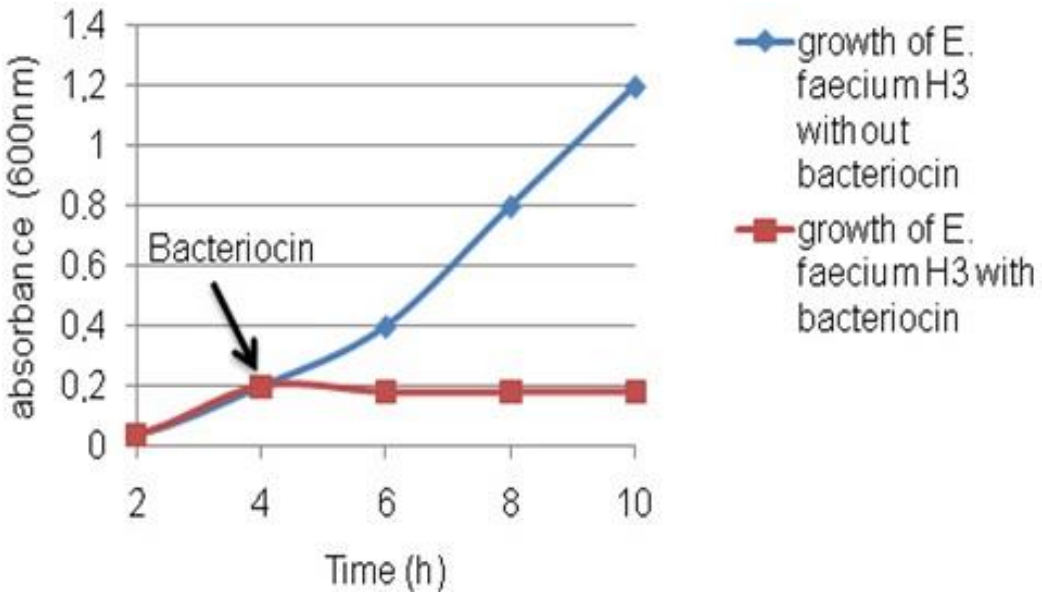

Figure 5. Growth of *Enterococcus faecium* H3 without bacteriocin GHB26 and with bacteriocin GHB26.

bacteriocin GHB26 was heat stable, sensitive to proteolytic enzymes, resistant to different pH values and to several chemical agents. For bacteriocin GHB26 purification, a four-step method including ammonium sulfate precipitation, Sephadex G-25 gel filtration chromatography, cation exchange chromatography on CM-Sephadex cellulose and RP-HPLC allowed the obtention of a pure bacteriocin, with a molecular weight close to 3.5 kDa. These results indicate that the bacteriocin GHB26 could be classified in the class II of bacteriocins. Bacteriocin GHB26 exhibited an inhibitory effect against *E. faecium* a frequent contaminant of milk and grains in our region. Based on these interesting characteristics, bacteriocin GHB26 could be a promising biopreservative in food.

Conflict of Interests

The authors have not declared any conflict of interests.

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REFERENCES

Aktypis A, Kalantzopoulos G (2003). Purification and characterization of thermophilin ST-1, a novel bacteriocin produced by *Streptococcus thermophilus* ACA-DC 0001. Lait. 83:365-378.

Altuntas EG **(**2013). Bacteriocins: A natural way to combat with pathogens. *In*: Mendez–Vilas A (eds) Microbial pathogens and strategies for combating them: science, technology and education, Turkey. pp. 1005-1015.

Balla E, Dicks LMT, Du Toit M, Van Der Merwe MJ, Holzapfel WH (2000). Characterization and cloning of the genes encoding enterocin 1071A and enterocin 1071B, two antimicrobial peptides produced by *Enterococcus faecalis* BFE 1071. Appl. Environ. Microbiol. 66:1298-1304.

Cintas LM, Casaus PH, Varstein LS, Hernandez PE, Nes IF (1997). Biochemical and genetic characterization of enterocin P, a novel *sec*-dependent bacteriocin from *Enterococcus faecium* P13 with a broad antimicrobial spectrum. Appl. Environ. Microbiol. 63:4321-4330.

Collins JW, La Ragione RM, Woodward MJ, Lsearle EJ (2009). Application of Prebiotics and Probiotics in Livestock. *In*: Charalampopoulos D, Rastall RA (Eds) Prebiotics and Probiotics Science and Technology, Springer Science+Business Media B.V., New York, USA. pp.1123-1192.

Cotter PD, Hill C, Ross RP (2005). Bacteriocins: developing innate immunity for food. Nat. Rev. Microbiol. 3:777-788.

Dellali A (2012). Lipolytic Activities of Lactic Acid Bacteria. Magister Dissertation, University of Oran 1, Oran, Algeria.

De Vuyst L, Leroy F (2007). Bacteriocins from lactic acid bacteria: Production, purification, and food applications. J. Mol. Microbiol. Biotechnol. 13:194-199.

Elegado FB, Kim WJ, Kwon DY (1997). Rapid purification, partial characterization and antimicrobial spectrum of the bacteriocin Pediocin ACM from *Pediococcus acidilactici* M. Int. J. Food. Microbiol. 37:111.

Graciela M, Vignolo M, de Kairuz M, Aida AP, de Ruiz H, Oilver G (1995). Influence of growth conditions on the production of lactocin 705, a bacteriocin produced by *L. casei* CRL 705. J. Appl. Bacteriol. 78:5-10.

Gürakan GC (2007). Biopreservation by lactic acid bacteria. In: Metabolism and Applications of Lactic acid bacteria, 15-31 ISBN: 978-81-308-0203-9 Editor: Barbaros Ozer. Published by Research Signpost, India.

Hernandez D, Cardell E, Zarate V (2005). Antimicrobial activity of lactic acid bacteria isolated from Tenerife cheese: initial characterization of plantaricin TF711, a bacteriocin-like substance produced by *Lactobacillus plantarum* TF711. J. Appl. Microbiol. 99:77-84.

Javed I (2009). Characterization of bacteriocin produced by lactic acid bacteria isolated from dairy products. PhD dissertation, University of Quaid-i-Azam , Islamabad, Pakistan.

Kawai Y, Saito T, Toba T, Samant SK, Itoh T (1994). Isolation and Characterization of a Highly Hydrophobic New Bacteriocin (Gassericin A) from *Lactobacillus gasseri* LA39. Biosci. Biotechnol. Biochem. 58:1218-1221.

Klaenhammer TR (1988). Bacteriocins of lactic acid bacteria. Biochimie. 70(3):337-349.

Kumar M, Ghosh N, Srivastava S (2009). Production and characterization of a bacteriocin produced by *Enterococcus faecium LR/6* . Internet. J. Microbiol. 8(1):13-14.

Lazreg L, Dalache F, Zadi-Karam H, Karam NE (2015). Bacteriocinogenic potential and genotypic characterization of three *Enterococcus faecium* isolates from Algerian raw milk and traditional butter. Afr. J. Biotechnol. 14(32):2517-2524.

Merzoug M, Dalache F, Zadi-Karam H, Karam NE (2015). Isolation and preliminary characterisation of bacteriocin produced by *Enterococcus faecium* GHB21 isolated from Algerian past e of dates “ghars”. Ann. Microbiol. 66:795-805.

Messaoudi S, Kergourlay G, Dalgalarrondo M, Choiset Y, Ferchichi M, Prévost H, Pilet MF, Chobert JM, Manai M, Dousset W(2012). Purification and characterization of a new bacteriocin active against Campylobacter produced by *Lactobacillus salivarius* SMXD51. Food. Microbiol. 32:129-134.

Ogunbanwo ST, Sanni AI, Onilude AA (2003). Characterization of bacteriocin produced by *Lactobacillus plantarum* F1 and *Lactobacillus brevis* OG1. Afr. J. Biotechnol. 2(8):219-227.

Park SH, Itoh K, Fujisawa T (2003). Characteristics and Identification of Enterocins Produced by *Enterococcus faecium* JCM 5804T. J. Appl. Microbiol. 95(2):294-300.

Pinto AL, Fernandes M, Pinto C, Albano H, Castilho F, Teixeira P, Gibbs PA (2009).Characterization of anti-*Listeria* bacteriocins isolated from shellfish: Potential antimicrobials to control non-fermented seafood. Int. J. Food. Microbiol. 129(1):50-58.

Satish KR, Kanmani P, Yuvaraj N, Paari KA, Pattukumar V, Arul V (2011). Purification and characterization of enterocin MC13 produced by a potential aquaculture probiont *Enterococcus faecium* MC13 isolated from the gut of Mugil cephalus. Can. J. Microbiol. 57:993-1001.

Schägger H (2006). Tricine–SDS PAGE. Nat .Protoc.1(1):16-22.

Smaoui S, Elleuch L, Bejar W, Karray-Rebai I, Ayadi I, Jaouadi B, Mathieu F, Chouayekh H, Bejar S, Mellouli L (2009). Inhibition of Fungi and Gram-Negative Bacteria by Bacteriocin BacTN635 Produced by *Lactobacillus plantarum* sp. TN635. Appl.Biochem. Biotechnol.162(4):1132-1146.

Srinivasan R, Kumawat DK, Kumar S, Saxena AK (2012).Purification and characterization of a bacteriocin from *Lactobacillus rhamnosus* L34. Ann. Microbiol. pp. 387-392.

Šušković J, Kos B, Beganović J, Leboš Pavunc A, Habjanič K, Matošić S (2010). Antimicrobial activity the most important property of probiotic and starter lactic acid bacteria. Food. Technol. Biotechnol. 48:296-307.

Tagg JR, McGiven AR (1971). Assay system for bacteriocins. J. Appl. Microbiol. 21:943-945.

Todorov SD, Wachsman M, Tomé E, Dousset X, Destro MT, Dicks LMT, Franco BDGM, Vaz-Velho M, Drider D (2010). Characterisation of an antiviral pediocin-like bacteriocin produced by *Enterococcus faecium*. Food. Microbiol. 27:869-879.

Vera Pingitore E, Salvucci E, Sesma F, Nader-Macias ME (2007). Different strategies for purification of antimicrobial peptides for Lactic Acid Bacteria (LAB). *In*: Mendez–Vilas A (eds). Commun. Curr. Res. Educ.Topics Trends Appl. Microbiol. Argent. 1:577-568.

Verdon J, Falge M, Maier E, Bruhn H, Steinert M, Faber C, Benz R, Héchard Y (2009). Detergent-like activity and α-helical structure of warnericin RK, an anti-Legionella peptide. Biophys. J. 97:1933-1940.

Von Mollendorff JW, Todorov SD, Dick LMT (2006). Comparison of bacteriocins produced by Lactic Acid Bacteria isolated from Boza, a cereal–based fermented beverage from the Belkan Peninsula. Curr. Microbiol. 53:209-216.

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Full Length Research Paper

β-Fructofuranosidase production by *Aspergillus versicolor* isolated from Atlantic forest and grown on apple pomace

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This study explores the production and characterization of an extracellular β-fructofuranosidase (FFase-I) by *Aspergillus versicolor* newly isolated from Atlantic Forest-Brazil. The β-fructofuranosidase production by fungus, after the optimization process using central composite design and response surface methodology, showed that 3% (w/v) apple pomace, an initial pH 7.5, and 12 days of cultivation provided the best conditions. The β-fructofuranosidase (FFase-I) was purified from the crude extract by 75% $(NH_4)_2SO_4$ precipitation followed by DEAE-Sephadex, and the molecular mass of the FFase-I was estimated to be 75 kDa by SDS-PAGE. Furthermore, the enzyme exhibited unusual tolerance to Cu^{2+}, sodium dodecyl sulfate (SDS), Tween 80, ethanol and acetone. The purified enzyme had an optimum pH of 6.0 and was stable over an acidic pH range of 3.0–6.0. The optimum temperature of the FFase-I was 55°C but was stable at 60°C for 7 h. Thus, the β-fructofuranosidase *A. versicolor* which has thermal stability and activity under acidic conditions would have potential application in sugar cane molasses hydrolysis for subsequent ethanol production.

Key words: *Aspergillus,* factorial design, fruit wastes, invertase.

INTRODUCTION

β-Fructofuranosidase (EC. 3.2.1.26), or invertase, catalyzes the irreversible hydrolysis of β-1,2-sucrose to

produce an equal mixture of glucose and fructose (Klotz and Campbell, 2004). This enzyme has been grouped in the glycoside hydrolase (GH) 32 family according to Carbohydrate-Active enZYmes Database (Lombard et al., 2014). This group of enzymes, together with the GH 68 family, is included in the GH-J clan. This family includes enzymes that catalyze the release of β-fructose from the non-reducing termini of various β-D-fructofuranoside substrates, such as invertases or β-fructofuranosidases; inulinases and levanases, which act on sucrose; raffinose; and inulin and levans, respectively (Álvaro-Benito et al., 2007; Kadowaki et al., 2013). Additionally, some β-fructofuranosidase enzymes have fructosyltransferase activity, resulting in the production of fructooligosaccharides (FOS), which are used commercially as prebiotics due to their physiological properties (Álvaro-Benito et al., 2007). This enzyme is also used extensively in lactic acid production, the fermentation of cane sugar molasses into ethanol, and calf feed production (Gehlawat, 2001; Rashad and Nooman 2009). β-Fructofuranosidase is also employed in the pharmaceutical industry in digestive aid tablets and powdered milk for infant food (Uma et al., 2012; Andrades et al., 2015). β-Fructofuranosidases from many microorganisms have been studied, including *Saccharomyces cerevisiae* (Andjelkovic et al., 2012), *Pichia anomala* (Rodriguez et al., 1995), *Aspergillus ochraceus* (Guimarães et al., 2007), *Aspergillus niveus* (Guimarães et al., 2009) and *Aspergillus niger* (Madhan et al., 2010).

Enzyme production through bioprocess technology is influenced by several variables, such as temperature, pH, cultivation time, and carbon and nitrogen sources present in the medium. Thus, optimization of culture conditions for enzyme production using a statistical approach is one of the first steps to develop a low-cost fermentation process. One of the advantages of this method is the possibility of observing the interactions between the variables and their effects on the response (Cui and Zhao, 2012).

In recent years, several lignocellulosic substrates have been explored in bioprocess technology for enzyme production or different bioactive molecules of biotechnological interest. The use of agro-industrial wastes as an alternative carbon source can reduce the cost of enzyme production by microorganisms. In general, sucrose has been reported in most studies as the best inducer for β-fructofuranosidase (FFase) production, but the use of agricultural residues, municipal solid wastes, and different sources of lignocellulosic biomass could reduce the cost of FFase production (Hayashi et al., 1992). Thus, investigations with unconventional substrates or agro-industrial waste using statistical tools can lead to the development of low cost bioprocessing applications. Hence, in this study, the apple pomace, a byproduct generated from fruit-processing industries, was investigated as a carbon source for β-fructofuranosidase production.

Brazil is among the nine biggest apple producers in the world, with a production of around 1.3 million tons of apples in 2011 (MAPA, 2013); the Brazilian apple production sector provides the raw material for juice and wine processing, releasing a significant amount of apple pomace (20–35% of the production). Generally, this waste is discharged into the soil as organic fertilizer or used as animal feed.

However, apple pomace is an interesting raw material, and it has attracted considerable attention as a potential source of sugar, dietary fiber, pectin, and citric acid flavoring, including for biotechnological applications (Canteri et al., 2012). *Aspergillus versicolor* has been reported to degrade compounds in environmental pollutants (Zhao et al., 2005) and to be a producer of xylanolytic enzyme complexes (Carmona et al., 1998). Thus, a central composite rotational design (CCRD)–based response surface methodology (RSM) approach was used to optimize cultivation conditions. Furthermore, a partial purification and characterization of an extracellular β-fructofuranosidase from *A. versicolor* isolated from the Atlantic Forest biome-Brazil was performed.

MATERIALS AND METHODS

Strain isolation and identification

A. versicolor was isolated from decaying plants obtained from the Bela Vista Biological Refuge in Foz do Iguaçu, Paraná, Brazil. Strain identification was based on an analysis of the internal transcribed spacer (ITS) regions of the ribosomal DNA gene (White et al., 1990). Genomic DNA of the fungus was extracted from mycelium obtained from submerged fermentation supplemented with 1% (w/v) glucose after 30 h of culture. The mycelium was collected by centrifugation (5,000 × *g*, 10 min), and the pellet obtained was then used for DNA extraction using the hexadecyltrimethylammonium bromide method. The primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3' forward) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3' reverse) were used to amplify the ITS1, ITS2, and 5.8S regions (White et al., 1990). The sequence determined was compared with other fungi sequences deposited in the National Center for Biotechnology Information (NCBI) databank using the Basic Local Alignment Tool (BLAST; http://www.ncbi.nlm.nih.gov). A neighbor-joining phylogenetic tree was constructed from the sequence of the ITS regions of *A.*

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Table 1. Experimental design matrix and results of β-fructofuranosidase production by *A. versicolor*

Run	Variables			Enzymatic activity (U mL^{-1})	
	X1 Apple pomace (%)	X2 Cultivation time (day)	X3 Initial pH	Experimental	Predicted
1	1 (-1)	4 (-1)	4.5 (-1)	0.50	3.31
2	3 (1)	4 (-1)	4.5 (-1)	1.37	0.03
3	1 (-1)	12 (1)	4.5 (-1)	23.83	23.93
4	3 (1)	12 (1)	4.5 (-1)	64.75	65.76
5	1 (-1)	4 (-1)	7.5 (1)	1.08	8.52
6	3 (1)	4 (-1)	7.5 (1)	2.25	5.25
7	1 (-1)	12 (1)	7.5 (1)	26.58	29.14
8	3 (1)	12 (1)	7.5 (1)	77.92	70.97
9	0.32 (-1.68)	8 (0)	6 (0)	0.75	-5.89
10	3.68 (1.68)	8 (0)	6 (0)	22.92	26.50
11	2 (0)	1 (-1.68)	6 (0)	1.50	-10.15
12	2 (0)	15 (1.68)	6 (0)	65.00	62.38
13	2 (0)	8 (0)	3.5 (-1.68)	37.33	36.84
14	2 (0)	8 (0)	8.5 (1.68)	48.17	45.60
15	2 (0)	8 (0)	6 (0)	21.67	26.11
16	2 (0)	8 (0)	6 (0)	23.55	26.11
17	2 (0)	8 (0)	6 (0)	26.51	26.11
18	2 (0)	8 (0)	6 (0)	20.95	26.11

versicolor using MEGA 6 (Tamura et al., 2013).

Culture conditions

A. versicolor was grown in 125-mL Erlenmeyer flasks containing 25 mL of sterile mineral medium comprised of the following (g/L): $NaNO_3$, (3), KH_2PO_4, (1), $MgSO_4.7H_2O$ (0.5), KCl (0.5), $Fe_2(SO_4)_3$ (0.01) and yeast extract (1), supplemented with apple pomace at different concentrations. The initial pH of the medium ranged from 3.5 to 8.5 and was adjusted as shown in the experimental design CCRD- 2^3 (Table 1). The apple pomace, used as carbon source, was obtained from local markets in Cascavel, Paraná, Brazil. The fungal spores were inoculated (10^5 conidia ml^{-1}), and cultures were incubated under static conditions at 28°C for varying times (Table 1). The submerged cultures were harvested under vacuum filtration using Whatman filter paper No. 1. The supernatant obtained was used as source of extracellular β-fructofuranosidase.

β-Fructofuranosidase production from *A. versicolor* by experimental design

A CCRD 2^3 was performed using three independent variables (initial pH of the medium, cultivation time and apple pomace concentration). The CCRD contained a matrix with 18 experimental runs, which included eight runs for factorial design, indicated by lowest (-1) and highest (+1) levels, six runs for axial points (two for each variable, [-1.68] and [+1.68]) and four replicates at the central point (0). The β-fructofuranosidase activity from *A. versicolor* was determined as response (Table 1). The mathematical relationship of response *Y* and the three variables X was determined by polynomial model Equation 1:

$$Y=\beta_0+\beta_1x_1+\beta_2x_2+\beta_3x_3+\beta_{12}x_1x_2+\beta_{13}x_1x_3+\beta_{23}x_2x_3+\beta_{11}x_1^2+\beta_{22}x_2^2+\beta_{33}x_3^2 \quad (1)$$

Where, Y represents the predicted response; β_0 is the intercept; β_1, β_2 and β_3 are the linear coefficients; β_{11}, β_{22} and β_{33} are the quadratic coefficients and β_{12}, β_{13} and β_{23} are the interaction coefficients. The data obtained from response surface methodology (RSM) for the β-fructofuranosidase production were subjected to analysis of variance (ANOVA) using the statistical software Statistica, v. 10. The accuracy and general ability of the above polynomial model were evaluated using the determination coefficient (R^2).

Enzyme assay and Protein quantification

The β-fructofuranosidase activity was determined using 200 mM sucrose as the substrate in sodium phosphate buffer (100 mM, pH 6.0) incubated with the extract for 10 min at 60°C. The amounts of reducing sugars were determined using dinitrosalicylic acid, according to Miller (1959). One unit of enzyme activity was defined as the amount of enzyme necessary to produce 1 µmol of glucose per minute under the assay conditions.

The protein was quantified using the Bradford (1976) method using bovine serum albumin as a standard, and absorbance of 280 nm was used for monitoring the protein in the column eluates.

Extracellular β-fructofuranosidase purification from *A. versicolor*

The crude extracts of *A. versicolor* were concentrated with $(NH_4)_2SO_4$ at 75% (w/v) and centrifuged at 5,478 x*g* for 30 min at 4°C. Then, the pellet was resuspended in Tris-HCl buffer (20 mM, pH 7.5) and dialyzed against the same buffer for 18 h at 4°C, loaded onto a DEAE-Sephadex chromatographic column (10 × 2.0 cm) and eluted using a linear gradient of NaCl (0 - 1.0 M). Three-milliliter fractions were collected at flow rate of 1 mL min^{-1}, and those with the highest β-fructofuranosidase activity were pooled, dialyzed overnight using distilled water at 4°C, lyophilized and used for biochemical characterization.

SDS-PAGE and zymogram

FFase-I from DEAE-Sephadex was analyzed using 10% SDS-PAGE, according to Laemmli (1970). The gel was stained with Coomassie blue R250 using the molecular mass markers (GE®), consisting of phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). Zymogram was obtained using the same SDS-PAGE procedure and stained according to methodology of Rehm et al. (1998). The gel was soaked for 30 min in Triton X-100 (0.5 %) at room temperature, rinsed with distilled water and incubated in 0.3 M sucrose dissolved in phosphate buffer (50 mM, pH 6.0) for 30 min at 40°C. Then, the gel was stained with 1% triphenyltetrazolium chloride dissolved in 0.25 M NaOH and incubated at 100°C. Acetic acid (5% solution) was added to stop the enzymatic reaction.

Effect of pH and temperature on enzyme activity and stability

The effect of pH was evaluated under standard assay conditions with 0.2 M sucrose dissolved in different pH values ranging from 3.0 to 7.5 of McIlvaine buffer. pH stability was determined by measuring the residual activity after the enzyme maintained for 48 h in different range of McIlvaine buffer (3.0, 4.0, 5.0 and 6.0).

The effect of temperature on the FFase-I activity was determined by performing a standard assay at temperature ranging from 40 to 80°C. In the thermal inactivation experiments, the enzyme was incubated at different temperatures (40, 50, 55, 60, 65 and 70°C) for up to 12 h. Residual enzyme activities were determined under standard assay conditions. All of these assays were repeated three times, and the results are expressed as relative percentages when compared with the highest value.

Effects of metal ions and chemical reagents on FFase-I activity

The effects of the following salts on FFase-I activity were tested: KCl, $MgSO_4$, NaCl, $SnCl_2$, $BaCl_2$, $(NH_4)_2SO_4$, $CaCl_2$, $HgCl_2$, $CuSO_4$ and $FeSO_4$. Additionally, the effects of the following compounds on FFase-I activity was tested: EDTA, sodium dodecyl sulfate (SDS), Tween 80, ethanol and acetone. The different compounds (1 mM or 5 mM) were pre-incubated for 15 min with the enzyme. After incubation, an aliquot was withdrawn and chilled on ice, and the hydrolytic activity was determined using the standard assay with sucrose as the substrate.

Kinetic parameters (K_m and V_{max}) determination

The kinetic parameters of the FFase-I were determined using sucrose as the substrate ranging from 5 to 70 mM. The K_m and V_{max} values were determined using the Lineweaver–Burk plot.

RESULTS AND DISCUSSION

Strain identification

The fungus was isolated from the Atlantic Forest biome in Brazil. The strain was identified both by its morphological characteristics and the sequencing of the ITS regions of the ribosomal DNA. The 508-bp fragment of the sequence showed 100% identity with other *A. versicolor* strains. This sequence was deposited in the GenBank of the National Center for Biotechnology Information (NCBI) under accession number KM396917. A phylogenetic tree was constructed based on the alignment of the sequences from the ribosomal genes from some *Aspergillus* species. This isolate (*Aspergillus versicolor* FT-5) is grouped with other *A. versicolor* strains (Figure 1).

Optimization of β-fructofuranosidase production using experimental design

The design matrix (cultivation time, carbon source, and initial pH) selected for the screening of the significant variables for β-fructofuranosidase production by *A. versicolor*, and the corresponding responses are shown in Table 1. Optimization of the culture conditions was carried out using CCRD 2^3 and RSM. Based on the previous results in the laboratory, apple pomace was chosen as the carbon source because it induces the production of β-fructofuranosidase in *A. versicolor* (unpublished data). In this study, CCRD showed that the best culture conditions for β-fructofuranosidase production by *A. versicolor* were 3% (w/v) apple pomace, 12 days of cultivation time, and an initial pH of 7.5, which resulted in 77.92 U mL^{-1} (Table 1). In addition, the enzyme production improved 150-fold between Run 1 and 8 (Table 1).

Cost-efficient industrial production of commercially important enzymes largely depends on cheap media as well as inexpensive substrates. Agro-industrial byproducts, such as sugar cane molasses and soybean meal, and other wastes have been reported as carbon sources for the production of invertases (Ohara et al., 2015).

Several studies describe the production of β-fructofuranosidase by fungi of the genus *Aspergillus* using agro-industrial waste or alternative low-cost, renewable carbon sources. For example, *A. niveus* was used to produced extracellular invertase (20 U mL^{-1}) with bagasse sugarcane supplemented with sucrose and glucose as the carbon sources (Guimarães et al*.,* 2009).

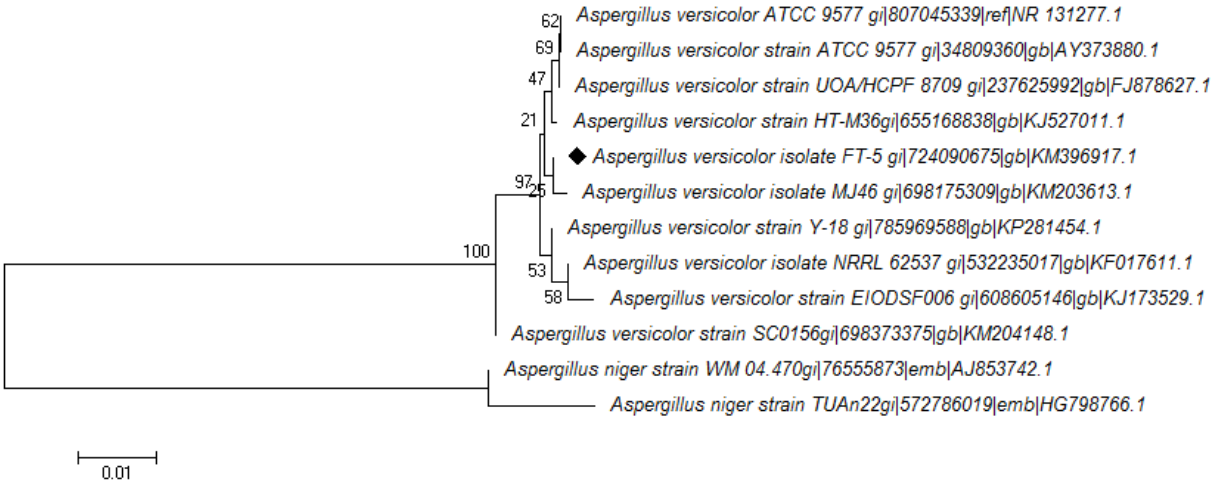

Figure 1. Phylogenetic tree constructed from sequences of the ITS regions of the ribosomal DNA from *A. versicolor* FT-5 and compared to sequences of other *A. versicolor* strains obtained from the NCBI GenBank database. *Aspergillus niger* strain WM04.47 and *A. niger* strain TUAn22 were used as out-groups. Fungus species is in bold symbol followed by Genbank accession number.

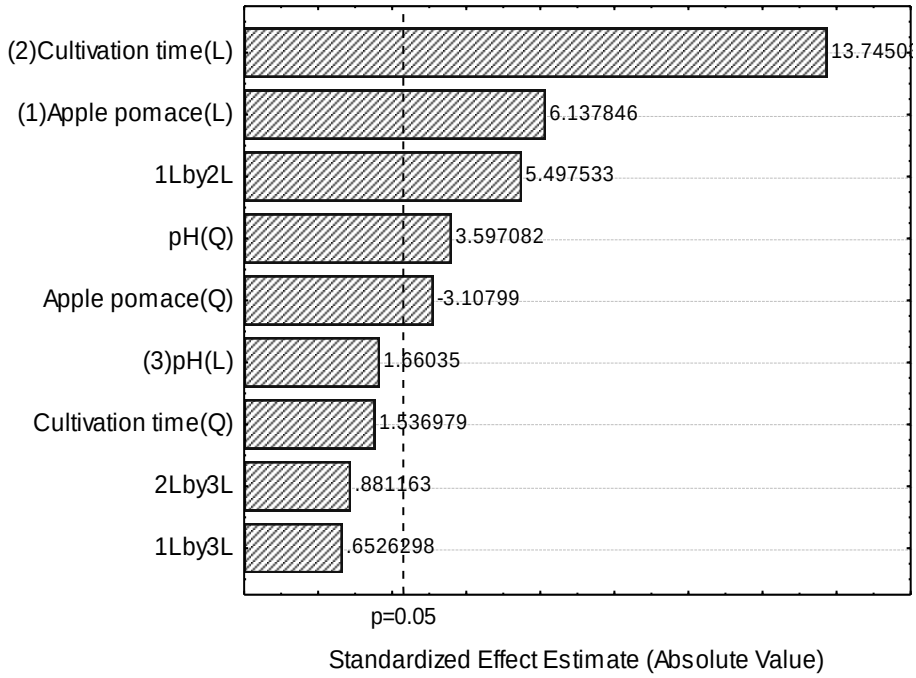

Figure 2. Pareto chart of the variable effects on β-fructofuranosidase activity using 2^3 factorial design.

Reddy et al. (2010) also reported the production of β-fructofuranosidase by *A. niger* PSSF21 using sugarcane molasses as the carbon source (19.1 U mL^{-1}).

The variables that most influenced β-fructofuranosidase production in this study were the apple pomace concentration (linear and quadratic term), cultivation time (linear term), and pH (quadratic term), which all had p values <0.05, as shown in Figure 2. The apple pomace

Table 2. Coefficient estimates by the regression model.

Parameter	Regression	Standard error	t(8)	p
Mean/Interc.	23.416	2.896	8.085	0.000 **
(1) Apple pomace (L)	9.640	1.570	6.138	0.000 **
Apple pomace (Q)	-5.077	1.634	-3.108	0.014 **
(2) Cultivation time (L)	21.587	1.571	13.745	0.000 **
Cultivation time (Q)	2.511	1.634	1.537	0.163
(3) pH (L)	2.607	1.571	1.660	0.135
pH (Q)	5.877	1.634	3.597	0.007 **
1L by 2L	11.276	2.051	5.497	0.001 **
1L by 3L	1.338	2.051	0.653	0.532
2L by 3L	1.807	2.051	0.881	0.404

**Statistically significant at 95% of confidence level.

Table 3. ANOVA of variable effects of β-fructofuranosidase production.

Source	Sum of squares	Degree of Freedom	Mean Square	F-value	F-listed	p-value
Regression	9497.20	6	1582.87	44.73	3.09	<0.001
Residual	389.22	11	35.38			
Lack of fit	370.79	8	46.35	7.54	8.85	0.062
Pure error	18.43	3	6.14			
Total	9886.42	17				

Determination coefficient (R^2= 0.9606).

concentration and cultivation time had a significant impact on the response (Table 2). Regression analysis of The experimental data yielded the following quadratic equation for β-fructofuranosidase production:

$$Y = 23.41+9.63X_1 -5.08 X_1^2 + 21.59 X_2 + 2.6 X_3 +5.87 X_3^2 +11.27 X_1 X_2 \quad (2)$$

Where, *Y* is the β-fructofuranosidase activity (U mL^{-1}), X_1 is the apple pomace concentration (%), X_2 is cultivation time (day), and X_3 is the initial pH of the medium.

Based on the F-test, the calculated F-value (44.73) is 14.47-fold higher than the critical F_{listed} (3.09) with $p<0.05$; this result implies a satisfactory representation of the process by the model. The lack of fit was not significant because the F_{cal} was lower than F_{listed}, indicating that the experimental data fit the model obtained (Table 3). The determination coefficient R^2 (0.9606) suggested that the fitted model could explain 96.06% of the total variation. Therefore, this study shows that the β-fructofuranosidase production from *A. versicolor* is enhanced when the cultivation time and apple pomace concentration are higher (Figure 3a). Likewise, higher values of initial pH and a longer cultivation time resulted in increased enzyme production (Figure 3b). Therefore, the importance of optimizing the production of invertase from fungus using an experimental design were also reported for the β-fructofuranosidase by *Aspergillus niger* in submerged and solid-state fermentation (Ashokkumar et al., 2001). Similarly, Driouch et al. (2010) also reported on optimized bioprocess for the production of fructofuranosidase by recombinant *A. niger* SKAn1015.

Purification of β-fructofuranosidase

β-Fructofuranosidase from *A. versicolor* was partially purified, and two pools were separated and named FFase-I (β-fructofuranosidase-I) and FFase-II (β-fructofuranosidase-II) (Figure 4). The ability to produce β-fructofuranosidase isoforms has been reported for other fungi, such as *Aureobasidium pullullans* DSM2404, which produced five β-fructofuranosidase isoforms grown in sucrose (Yoshikawa et al., 2006). In addition, *Fusarium oxysporum* produced two extracellular isoforms of this

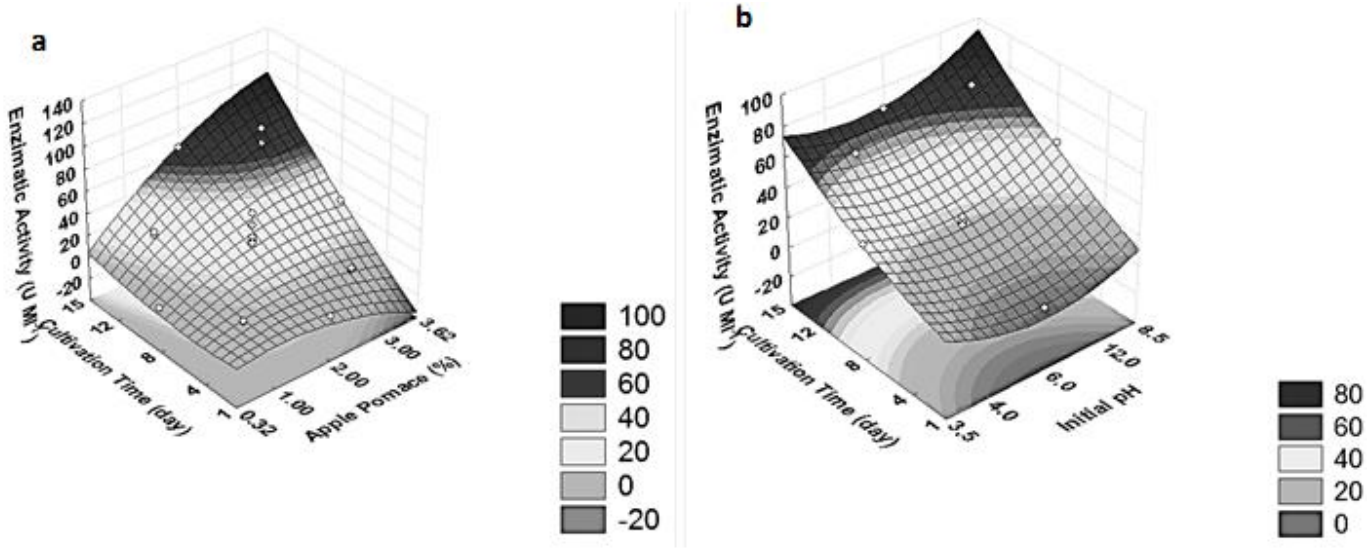

Figure 3. Three-dimensional plot representing effects of cultivation time and apple pomace on β-fructofuranosidase production by *A. versicolor* (a); cultivation time and initial pH (b).

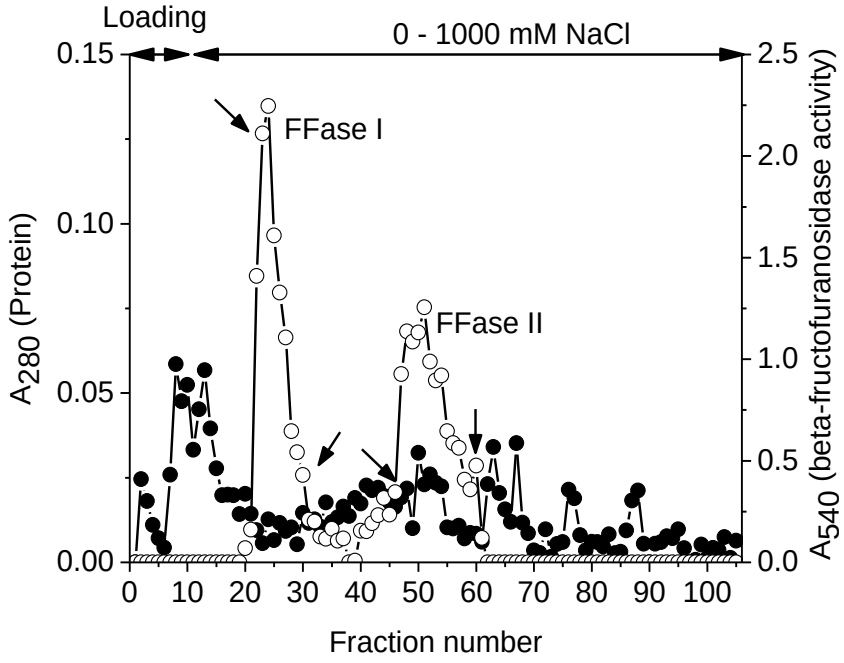

Figure 4. Chromatographic profile of β-fructofuranosidase activity from *A. versicolor* using a DEAE-Sephadex column equilibrated with Tris-HCl buffer (20 mM, pH 7.5) and eluted using a NaCl gradient. (○) absorbance 540 nm; (●) absorbance 280 nm.

enzyme using fructose as the carbon source (Wolska-Mitaszko et al., 2007). In this study, the FFase-I was purified with a yield of 22.57% after DEAE-Sephadex chromatography (Table 4). Zymogram revealed the presence of a zone of hydrolysis that corresponded with the Coomassie-stained band of purified beta-

Table 4. Summary of the purification of extracellular FFase-I from *A. versicolor.*

Step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Yield (%)	Purification fold
Crude extract	37,714.29	13,258.15	2.84	100.00	1.00
$(NH_4)_2SO_4$ precipitation	15,748.57	2932.60	5.37	41.76	1.89
DEAE Sephadex	8,510.91	815.74	10.43	22.57	3.67

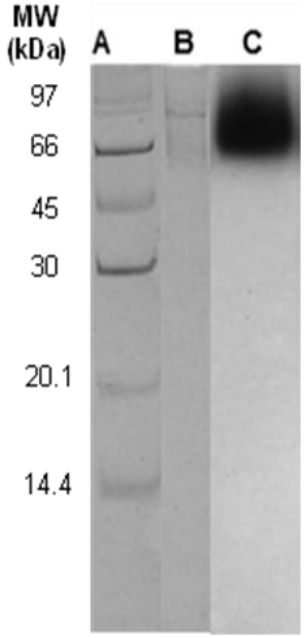

Figure 5. SDS-PAGE and zymogram of FFase-I produced by *A. versicolor*. a) Molecular mass marker; b) FFase-I after DEAE-Sephadex; c) zymogram.

fructofuranosidase-I; the molecular mass of the FFase-I was estimated to be 75 kDa by SDS-PAGE analysis (Figure 5). Some *Aspergillus* genera showed β-fructofuranosidases with different molecular masses, including *A. ochraceus* (13 kDa [Gosh et al., 2001] and 13.5 kDa invertase [Guimarães et al., 2007]), *A. niger* (75 kDa invertase [Goosen et al., 2007]), and 60 kDa invertase from *A. japonicus* (Wang and Zhou, 2006).

Effect of temperature on enzyme activity and thermal stability

FFase-I was highly active between 55 and 60°C (Figure 6a), which was higher than the β-fructofuranosidases from *Mucor geophillus* (45°C) (Quershi et al., 2012) and *A. caespitosus* (50°C) (Alegre et al., 2009), but most of the fungi show the high optimal temperature, especially between 50 and 60°C.

The thermal stability of the FFase-I was 100% for 12 h when incubated at 40, 50 and 55°C. The half-life at 60°C was 7 h, and its activity was 40 and 26% for 8 and 12 h, respectively (Figure 6b). However, at 65 and 70°C, the half-life decreased to 30 min. The FFase-I from *A. versicolor* had higher thermal stability when compared with β-fructofuranosidases produced by *A. phoenicis*, which was stable at 50°C for 1 h (Rustiguel et al., 2010), and *A. caespitosus*, which was stable at 60°C for 1 h and 20 min (Alegre et al., 2009).

Effect of pH on enzyme activity and stability

The optimum pH for the activity of FFase-I from *A. versicolor* was 6.0 (Figure 7a). Similar values were observed with extracellular β-fructofuranosidases produced by *A. caespitosus* and *S. cerevisiae* NRRLY12632 (Alegre et al., 2009; Mona and Mohamed, 2009). Most fungal β-fructofuranosidases have an optimum pH in the acidic range, as observed for *A. phoenicis* (Rustiguel et al., 2010) and *C. sitophila* PSSF84 (Patil et al., 2011) (both pH 4.5), and *M. geophillus* (pH 5.0) (Quershi et al., 2012). The FFase-I from *A. versicolor* is stable for 12 h between pH 3 and 6 (Figure 7b). This β-fructofuranosidases' property with acidic pH range is commonly found in commercial invertase microbial sources.

Effect of metal ions and chemical reagents on FFase-I activity

Among the salts tested, Fe^{+2} (5 mM) increased the activity of FFase-I by 27.32%, suggesting that the ion acts as enzyme cofactor. Furthermore, the enzyme was tolerant to ethanol, acetone and detergents (SDS and Tween 80). Enzymes that are stable in the presence of compounds such as organic solvents, surfactants and metallic ions are considered relevant for catalysis in biotechnological applications. In addition, FFase-I was tolerant to the presence of copper, which is unusual for Most enzymes because they are often inhibited by

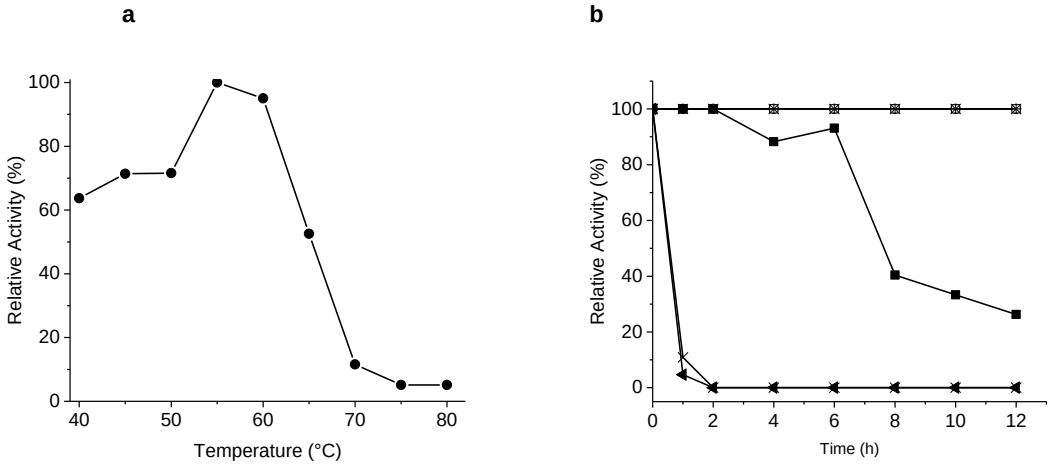

Figure 6. Effect of temperature on FFase-I activity (a) and the thermal stability of FFase-I (b) produced by *A. versicolor*; 40°C (○), 50°C (□), 55°C (*), 60°C (■), 65°C (X), 70°C (◄).

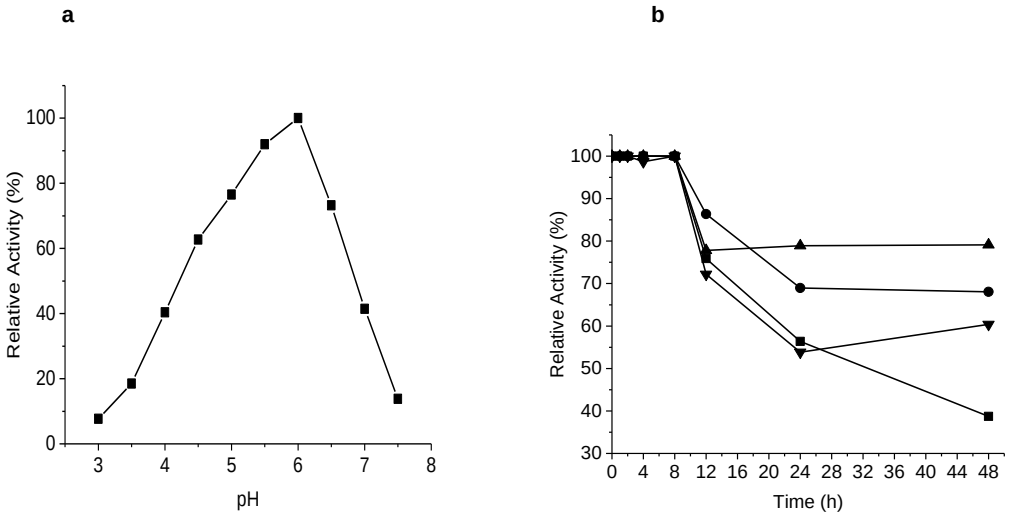

Figure 7. Influence of pH on the activity of FFase-I (a) and the thermal stability of FFase-I (b) produced by *A. versicolor* pH 3.0 (■), pH 4.0 (●), pH 5.0 (▲), pH 6.0 (▼).

copper (Guimarães et al., 2007; Uma et al., 2012). In contrast, Hg^{2+} strongly inhibited the enzyme activity at both concentrations tested (1 and 5 mM) by 86–89% (Table 5). β-Fructofuranosidases from *A. niger* IMI303386 (Nguyen et al., 2005), *Chrysonilia sitophila* PSSF84 (Patil et al., 2011) and *A. phoenicis* (Rustiguel et al., 2010) were also strongly inhibited by Hg^{2+}.

Kinetic properties

The apparent K_m and V_{max} values for the FFase-I from *A. versicolor* were 26.71 mM and 56.98 U mg^{-1}, respectively, using sucrose as the substrate. These results were very similar to those obtained with β-fructofuranosidase from *A. phoenicis,* which had K_m and V_{max} values of 25 mM and 55.679 U mg^{-1}, respectively (Rustiguel et al., 2010). However, the K_m from β-fructofuranosidase from *A. versicolor* was higher (lower affinity for the substrate) than that of *A. ochraceus*, which was 13.4 mM (Guimarães et al., 2007), as well as *A. niveus*, which was 5.78 mM (Guimarães et al., 2009).

Conclusions

β-Fructofuranosidase produced by *A. versicolor* isolated from the Parana Atlantic Forest biome showed a

Table 5. Effect of metal ions and chemical reagents on FFase–I activity.

Compound	β-Fructofuranosidase activity (%)	
	1 mM	5 mM
KCl	107.33	111.69
$MgSO_4$	108.78	110.72
NaCl	103.46	106.64
$SnCl_2$	96.46	96.32
$BaCl_2$	100.21	104.84
$(NH_4)_2SO_4$	101.13	101.70
$CaCl_2$	111.00	105.74
$HgCl_2$	11.69	14.32
$CuSO_4$	105.09	99.15
$FeSO_4$	114.04	127.32
EDTA	94.63	92.93
Ethanol	100.28	94.06
Acetone	101.41	102.40
SDS	104.24	90.95
Tween 80	101.87	103.32
Control	100.00	100.00

significant improvement (150-fold) after optimized culture conditions using apple pomace as an inexpensive and alternative carbon source. The purified FFase-I was stable under acidic pH (3–6) and was thermally stable up to 60°C. In addition, the enzyme was also tolerant to different metallic ions, organic solvents (ethanol and acetone) and detergents (SDS and Tween 80). Therefore, the FFase-I presents promising properties and is suitable for biotechnological exploitation.

Conflict of Interests

The authors have not declared any conflict of interests.

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REFERENCES

Alegre ACP, Polizeli MLTM, Terenzi HF, Jorge JA, Guimarães LHS (2009). Production of thermostable invertase by *Aspergillus caespitosus* under submerged or solid state fermentation using agroindustrial residues as carbon source. Braz. J. Microbiol. 40:612-622.

Álvaro-Benito M, Abreu M, Fernández-Arrojo L, Plou FJ, Jiménez-Barbero J, Ballesteros A, Polaina J, Fernández-Lobato M (2007). Characterization of a β-fructofuranosidase from *Schwanniomyces occidentalis* with transfructosylating activity yielding the prebiotic 6-kestose. J. Biotechnol. 132:75-81.

Andjelkovic U, Theisgen S, Scheidt HA, Petkovic M, Huster D, Vujcic Z (2012). The thermal stability of the external invertase isoforms from *Saccharomyces cerevisiae* correlates with the surface charge density. Biochimie. 94:510-515.

Andrades D, Arfelli VC, Oriente A, Henn C, Pereira VAAC, Simão RCG, Silva JLC, Kadowaki MK (2015). Improved production of β-galactosidase and β-fructofuranosidase by fungi using alternative carbon sources. Sci. Res. Essays. 10:236-242.

Ashokkumar B, Kayalvizhi N, Gunasekaran P (2001). Optimization of media for β-fructofuranosidase production by *Aspergillus niger* in submerged and solid state fermentation. Process Biochem. 37:331-338.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Canteri MHG, Nogueira A, Petkowicz CLO, Wosiacki G (2012). Characterization of Apple Pectin – A Chromatographic Approach. InTech**.** Open Access Publisher.

Carmona EC, Brochetto-Braga MR, Pizzirani-Kleiner AA, Jorge JA (1998). Purification and biochemical characterization of an endoxylanase from *Aspergillus versicolor.* FEMS Microbiol. Lett. 166:311-315.

Cui F, Zhao L (2012). Optimization of xylanase Production from *Penicillium* sp.WX-Z1by a two-step statistical strategy: Plackett-Burman and Box-Behnken experimental design. Int. J. Mol. Sci. 13:10630-10646.

Driouch H, Roth A, Dersch P, Wittmann C (2010). Optimized bioprocess for production of fructofuranosidase by recombinant *Aspergillus niger*. Appl. Microbiol. Biotechnol. 87:2011-2024.

Gehlawat KJ (2001). New technology for invert sugar and high fructose syrups from sugarcane. Indian J. Chem. Technol. 8:28-32.

Goosen C, Yuan XL, Van Munster JM, Ram AF, Van Der Maarel MJ, Dijkhuizen L (2007). Molecular and biochemical characterization of a novel intracellular invertase from *Aspergillus niger* with transfructosylating activity. Eukaryot. Cell. 6:674-681.

Gosh K, Dhar A, Samanta TB (2001). Purification and characterization of an invertase produced by *Aspergillus ochraceus* TS. Indian J.

Biochem. Biophys. 38:180-185.
Guimarães LHS, Somera AF, Terenzi HF, Polizeli MLTM, Jorge JA (2009). Production of β-fructofuranosidases by *Aspergillus niveus* using agroindustrial residues as carbon sources: Characterization of an intracellular enzyme accumulated in the presence of glucose. Process Biochem. 44:237-241.
Guimarães LHS, Terenzi HF, Polizeli MLT, Jorge JA (2007). Production and characterization of a thermostable extracellular ß-D-fructofuranosidase produced by *Aspergillus ochraceus* with agroindustrial residues as carbon sources. Enzyme Microb. Technol. 42:52-57.
Hayashi S, Matsuzaki K, Takasaki Y, Ueno H, Imada K (1992). Production of β-fructofuranosidase by *Aspergillus japonicus*. World J Microbiol. Biotechnol. 8:155-159.
MAPA (2013). Ministry of Agriculture Livestock and Supply. 54 (6). Available at: http://www.agricultura.gov.br
Kadowaki MK, Simao RCG, Da Conceição Silva JL, Osaku CA, Guimaraes LHS (2013). Biotechnological advances in fungal invertases. In: Polizeli MLTM, Rai M (Ed.), *Fungal Enzymes* CRC Press Taylor & Francis Group, New York, pp.1-30.
Klotz KL, Campbell LG (2004). Sucrose catabolism in developing roots of three beta vulgaris genotypes with different yield and sucrose accumulating capacities. J. Sugar Beet Res. 41(3):73-88.
Lombard V, Golaconda RH, Drula E, Coutinho PM, Henrissat B (2014). The Carbohydrate-active enzymes database (CAZy) in 2013. Nucleic Acids Res. 42:490-495.
Madhan SSR, Sathyavani R, Niket B (2010). Production and partial purification of invertase using *Cympopogan caecius* leaf powder as substrate. J. Microbiol. 50:318-324.
Miller GL (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31:426-489.
Nguyen QD, Rezessy-Szabó JM, Bhat MK, Hoschke A (2005). Purification and some properties of β-fructofuranosidase from *Aspergillus niger* IMI303386. Process Biochem. 40:2461-2466.
Ohara A, Castro RJS, Nishide TG, Dias FFG, Bagagli MP, Sato HH (2015). Invertase production by *Aspergillus niger* under solid state fermentation: Focus on physical–chemical parameters, synergistic and antagonistic effects using agro-industrial wastes. Biocatal Agric Biotechnol. 4:645-652.
Patil PR, Reddy GSN, Sulochana MB (2011). Production, optimization and characterization of β-fructofuranosidase by *Chrysonilia sitophila* PSSF84 – A novel source. Ind. J. Biotech. 10:56-64.
Quershi AS, Khushk I, Bhutto MA, Dahot MU, Haq I, Bano S, Iqbal H (2012). Production and partial characterization of invertase from *Mucor geophillus* EFRL 03. Afr. J. Biotechnol. 11:10736-10743.
Rashad MM, Nooman MU (2009). Production, purification and characterization of extracellular *Saccharomyses Cerevisiae* NRRL Y-12632 by solid- fermentation of red carrot residue. Aust J. Basic. Appl. Sci. 3:1910-1919.
Reddy PP, Reddy GSN, Sulochana MB (2010). Highly thermostable β-fructofuranosidase from *Aspergillus niger* PSSF21 and its application in the synthesis of fructooligosacharides from agro industrial residue. Asian J. Biotechnol. 2:86-89.
Rehm J, Willmitzer L, Heyer AG (1998). Production of 1-kestose in transgenic yeast expressing a fructosiltransferase from *Aspergillus foetidus*. J Bacteriol. 180:1305-1310.
Rodriguez J, Perez JA, Ruiz T, Rodriguez L (1995). Characterization of the invertase from *Pichia anomala*. Biochem. J. 306:235-239.
Rustiguel CB, Terenzi HF, Jorge JA, Guimarães LHS (2010). A novel silver-activated extracelular β-D-fructofuranosidase from *Aspergillus phoenicis*. J. Mol. Catal. B: Enzym. 67:10-15.
Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol. Biol. Evol. 30:2725-2729.
Uma C, Gomathi D, Ravikumar G, Kalaiselvi M, Gopalakrishnan VK (2012). Production and properties of Invertase from a *Cladosporium cladosporioides* in SmF using pomegranate peel waste as Substrate. Asian Pac. J. Trop. Biomed. 2(2):S605-S611.
Wang L, Zhou H (2006). Isolation and identification of a novel *Aspergillus japonicus* JN19 producing beta-fructofuranosidase and characterization of the enzyme. J Food Biochem. 30:641-658.
White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ eds PCR Protocols:a guide to methods and applications. Academic Press, New York, USA. 315-322.
Wolska-Mitaszko B, Jaroszuk-Scisel J, Pszeniczna K (2007). Isoforms of trehalase and invertase of *Fusarium oxysporum*. Mycol. Res. 111:456-465.
Yoshikawa JS, Aamachi S, Shinoyama H, Fujii T (2006). Multiple β-fructofuranosidases by *Aureobasidium pullulans* DSM2404 and their roles in frutooligosaccharide production. FEMS Microbiol. Lett. 265:159-163.

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Molecular characterization of midgut bacteria of *Aedes albopictus* exhibiting swarming motility property

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***Aedes* mosquitoes are responsible for the transmission of arboviruses including dengue, chikungunya, zika, and yellow fever throughout the world. Mosquito gut harbors a variety of microbes, known to play a potential role in various physiological processes such as food digestion and pathogen development. Many bacterial species exert various movements like swarming, gliding and twitching which vary with individual bacterial species. Swarming is one of the movement patterns of bacterial cells in which newly growing cells direct themselves towards the edges of the colony on the solid agar plate. We have isolated a bacterial colony, which exerts swarming activity and moves away from its inoculation point following a translucent path. After repeated sub-culturing of the colony we isolated two different bacterial species which were found to be highly associated to each other in co-culture. Various techniques like biochemical analysis, 16S rRNA gene sequence based analysis, MALDI-TOFF MS and Scanning Electron Microscopy (SEM) were applied for in-depth characterization of isolates. The isolates were identified as *Staphylococcus saprophyticus* and *Brevibacillus agri.* Both bacterial isolates showed swarming activity in co-culture on the solid agar surface, but lost the activity when tested individually. Moreover, the swarming activity was even not recovered when both purified bacterial isolates were mixed at different concentrations. This demonstrated that an integral connection exists between the two species which is responsible for their swarming activity in co-culture.**

Key words: Swarming, midgut, microbiota, *Aedes albopictus*, pathogens.

INTRODUCTION

Many bacterial species exert various movements like swarming, gliding and twitching which varies with individual bacterial species. Various types of movement properties have been reported in many prokaryotic cells, mainly in bacteria (Cong et al., 2011; Fraser and Hughes, 1999; Harshey, 2003; Kim et al., 2003; Merino et al.,

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2006). The swimming movement pattern is the individual bacterial cells behavior, whereas, swarming is shown by bacterial colony (Cong et al., 2011). Many bacterial species have the characteristic to grow and spread on the surface such as nutrient agar or eukaryotic cells in a tissue, from where they take up the nutrient and water. Swarming is one of the movement patterns of bacterial cells in which new cells direct themselves towards the edges of the colony (Kaiser, 2007). In swarming motility, the bacterial cells required to get in touch with solid substrate and this interaction between bacterial cells and solid surface make them swarming proficient (Kearns, 2010). Swarming reduces competition between bacterial cells for the nutrient and speeds up colony growth (Kaiser et al., 2007). Some other surface phenomena feature like host invasion and biofilm formation has been shared in the swarming movement as in *Proteus mirabilis*, which facilitates urinary tract colonization and encouraging biofilm formation on catheters (Cong et al., 2011; Allison et al., 1994; Stickler et al., 1998).

Aedes aegypti and *Ae. albopictus* are the most important vectors of arboviruses including dengue, chikungunya, zika, and yellow fever throughout the world. Mosquito gut harbors a diverse variety of microbes, which interact with disease-causing parasites and influence vectorial capacity of mosquito vectors (Yadav et al., 2015a, b, Chandel et al., 2013; Moro et al., 2013; Minard et al., 2013; Dong et al., 2009, Rani et al., 2009). The midgut bacterial communities of *Anopheles gambiae* decrease the susceptibility to *Plasmodium falciparum* infection (Dong et al., 2009). However, it has been also reported that the presence of some bacterial species promote the *Plasmodium* infection (Boissière et al., 2012). The bacterial species *Serratia marcescens* enhances the susceptibility to dengue and chikungunya viral infection (Apte-Deshpande et al., 2012, 2014). Chikungunya viral infection changes the midgut bacterial diversity in *Ae. albopictus* and increases the bacterial genera of Enterobacteriaceae family and decreases *Wolbachia* and *Blattabacterium* (Boissière et al., 2012, Zouache et al., 2012, Minard et al., 2013).

The midgut microbiota of insects also interacts to each other either in cooperative or competitive manner (Minard et al., 2013). An interspecific competition was identified by bacterial species *Serratia marcescens* on *Sphingomonas* and Burkholderiaceae family members isolated from *Ae. aegypti* (Terenius et al., 2012). The bacterial diversity was observed low in mosquito's midgut in the presence of *S. marcescens* and the same result was found in the case of desert locust *Schistocerca gregaria* (Dillon et al., 2002). However, in *Ae. albopictus* a convincing association-ship was shown by two bacterial genera *Asaia* and *Acinetobacter* (Minard et al., 2013).

It has been demonstrated that bacterial species interact to each other inside the mosquito's midgut (Minard et al., 2013). However, the interspecific interaction between the bacterial isolates responsible for swarming motility was not described previously. The aim of this study was characterization of bacterial isolates, exhibiting swarming motility in co-culture isolated from the midgut of *Ae. albopictus*. The information about the midgut bacterial interaction and behavior is limited. Hence, the present study might be a step towards understanding the bacterial interaction of mosquito's midgut, which plays various important roles in defense, food digestion and pathogen development (Minard et al., 2013; Dong et al., 2009).

MATERIALS AND METHODS

Sample collection and isolation of midgut bacteria

Larvae and pupae of *Aedes* mosquitoes were collected from Missamari Military Farm, Sonitpur, North East India, and pupae were transferred to the cages covered with net for emergence. 24 h after emergence, female mosquitoes were removed and anesthetized with chloroform for confirming the species *Ae. albopictus* on the basis of morphological characteristics using taxonomic pictorial keys (Rueda, 2004). The mosquitoes were dissected for the isolation of midgut bacteria according to the protocol previously described by Chandel et al. (2013). The dissected midgut was transferred in the sterile Phosphate-buffered saline (PBS) and homogenized with sterilized micro-pestle. The homogenate was pour plated on nutrient agar and incubated at 37°C for 24 h (Chandel et al., 2013). The bacteria colony designated as MS1-8 was further serially diluted up to 10^{-8} and two morphological distinguished bacterial species designated as MS1-8-I and MS1-8-II was obtained.

Characterization of bacterial isolates

Phenotypic characterization

The isolated bacteria were characterized based on colony morphology (like shape, size, colour, margin, opacity, elevation) and Gram staining (Rani et al., 2009). Motility of individual bacterial species was studied using the hanging drop method under a light microscope (*Leica* DM E compound microscope). Biochemical tests such as nitrate reduction, urease activity, citrate utilization, H_2S production, Voges-Proskauer (VP), Methyl Red (MR), β–galactosidase activity, ornithine and lysine utilization activity and carbon source utilization profile were also performed according to manufacturer's instruction (HiMedia kit).

Matrix-assisted laser desorption / ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis

The MALDI-TOF-MS identification system was used for the identification of MS1-8-I and MS1-8-II. The fresh bacterial culture was directly deposited on a MALDI-TOF MTP 96 target plate (Bruker Daltonics GmbH) and inactivated with 96% formic acid (1 µl) followed by overlaying 1 µl of matrix solution (α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, and 2.5% trifluoroacetic acid). Finally, proceeds with air-drying at room temperature for crystal formation of matrix-sample and spectra measured and recorded using Microflex III mass spectrometer (Bruker Daltonics) with a 337- nm nitrogen laser. The obtained raw spectra were compared with reference Biotyper database using Biotyper

Table 1. Restriction enzymes used for cutting 16S rRNA gene of all isolates with their cutting sites.

S/N	Restriction enzyme	Cutting site
1	MvaI	5' CC↓WGG 3' 3' GGW↑CC 5'
2	EcoRI	5' G↓AATTC 3' 3' CTTAA↑G 5'
3	SphI	5' GCATG↓C 3' 3' C↑GTACG 5'
4	MspI	5' C↓CGG 3' 3' GGC↑C 5'

software, *ver*. 3.0 (Prakash et al., 2014).

PCR amplification and sequencing of 16S rRNA gene

Genomic DNA was isolated from overnight grown bacterial culture, according to modified protocol of Sambrook et al. (2001). Ribosomal 16S RNA gene from genomic DNA was amplified according to the protocol described by Yadav et al. (2015 b) using an universal primer set 16S1 (5'-GAGTTTGATCCTGGCTCA-3') and 16S2 (5'-CGGCTACCTTGTTACGACTT-3') (Alam et al., 2006). PCR cleanup kit (Chromous Biotech Pvt. Ltd., Bangalore, India) was used for the purification of amplified PCR product followed by manufacturer's instruction and was sequenced on ABI 3500xL Genetic Analyzer (Applied Biosystems Inc. Foster City, CA) at Chromous Biotech Pvt. Ltd, Bangalore, India. Forward and the reverse sequence were aligned to get a complete sequence.

Sequence analyses

Obtained 16S rRNA gene sequences of bacterial isolates were analyzed using EzTaxon server (http://www.ezbiocloud.net/eztaxon) and BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and closely related sequences were recovered from GenBank database for phylogenetic analysis. Multiple sequence alignment was performed using the Clustal W and unaligned sequences at the end were trimmed out. MEGA 6.0 package was used to perform phylogenetic relatedness using neighbor joining and Kimura 2 distances parameter method with thousand bootstrap replicates (Tamura et al., 2013). The sequence of 16S rRNA gene of *Kocuria palustris* (Y16263) was used as out group. Sequences were submitted to GenBank under the accession numbers (KT315584 and KT315585).

Scanning electron microscopy (SEM)

Overnight broth culture of bacteria was centrifuged at 5000 rpm for 10 min followed by two times pellet washing with PBS. Glutaraldehyde (2.5% in PBS) was added to cell pellets, mixed gently and incubated at 4°C for 6 h for fixation. Supernatant was discarded after centrifugation followed by three times pellet washing with PBS. Bacterial specimens were dehydrated using gradually increased concentrations of ethanol (30, 50, 80 and 100%). While using 30, 50 and 80% ethanol, the ethanol was added to pellet, which was mixed, and incubated at room temperature for 10 min? It was then centrifuged and the supernatant was removed, but in case of 100% ethanol, samples were directly transferred to pre-sterilized cover slip and allowed to air dry. Dry samples were platinum coated and examined under (SEM) JEOL-JSM-6390LV Scanning Electron Microscope (Yadav et al., 2015b).

PCR-RFLP analysis of 16S rRNA gene

A total of 4 different restriction enzymes having different restriction sites (MvaI, EcoRI, SphI, and MspI) were used for the analysis of restriction fragment length polymorphism (RFLP) patterns of 16S rRNA gene of bacterial isolates (Table 1). Purified 16S rRNA PCR amplicons were used for the digestion through restriction enzymes according to the manufacturer's instruction (Thermo fisher scientific, UK). Restriction digested PCR amplicons were visualized on 2% agarose gel.

Movement property study using isolated bacterial species

Overnight grown bacterial cultures of both MS1-8-I and MS1-8-II were mixed in a gradually increasing proportion followed by inoculation on solid agar plates for checking the regaining of movement property. Prior to mixing, the optical density of both bacterial cultures was measured at 630 nm to find out the concentration of bacteria in each culture. The optical density of each bacterial culture was equally maintained and mixed in a specific proportion, that is, increasing the concentration of one culture (10, 20, 30, 40, 50, 60, 70, 80 and 90) and decreasing the concentration of another culture (90, 80, 70, 60, 50, 40, 30, 20 and 10). The mixed culture was inoculated on the agar plate at 0, 24 and 48 h intervals. Zero hour samples were inoculated immediately after mixing of the individual isolate, whereas, for 24 and 48 h mixed bacterial culture was incubated at 37°C at 120 rpm and inoculated on the agar plate after 24 and 48 h. The inoculated plates were incubated at 37°C for 12 to 24 h and movement properties of bacterial colonies were observed.

Both the overnight broth cultures MS1-8-I and MS1-8-II were mixed on the solid agar plate surface. For this purpose, one culture was inoculated onto agar plate through spreader and was incubated at 37°C for 3 to 4 h. Later on, the other culture was point inoculated in the center of the lawn of first culture and incubated at 37°C for 24 to 48 h. This procedure was followed for the both bacterial species, respectively.

RESULTS

The bacterial isolate MS1-8 was isolated from the midgut of an Asian tiger mosquito *Ae. albopictus*. Initially, MS1-8 appeared as a single bacterial colony on a solid agar plate but repeated sequencing of 16S rRNA gene revealed the presence of mixed sequences. Further, microscopic examination under a light microscope, Gram staining and Scanning Electron Microscopy (SEM) demonstrated that the colony designated as MS1-8 was a mixture of two different type of bacterial isolates (round *vs.* rod) which were characterized by using various techniques.

Phenotypic characterization

Phenotypic characteristics of bacterial isolates were

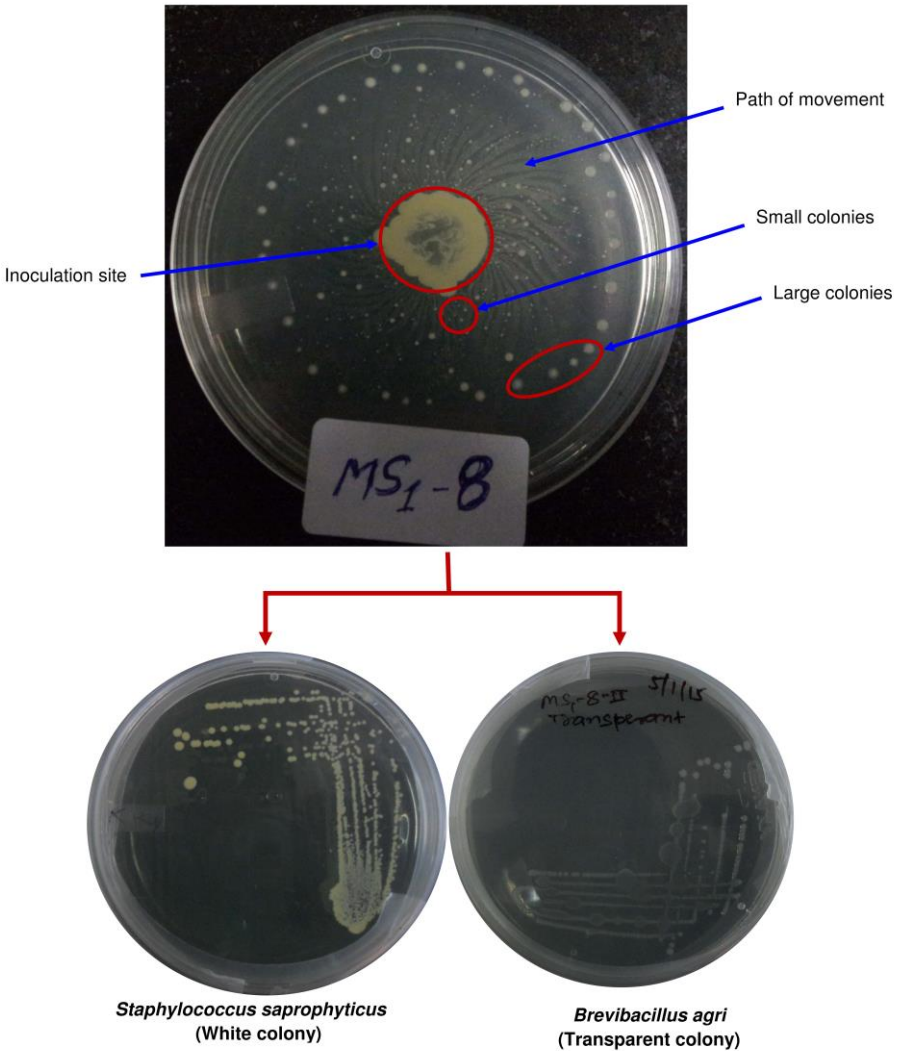

Figure 1. Bacteria colony of MS1-8 exhibiting movement property: I. Small colonies of MS1-8 appeared near to inoculation site and large colonies at peripheral site and movement of colonies follows a transparent pathway. II. White purified bacterial colonies MS1-8-I. III. Transparent purified bacterial colonies MS1-8-II.

initially studied for the first line of characterization. The isolated parental bacterial colony MS1-8 showed swarming property when inoculated on the center of nutrient agar plates and left their path from the inoculation site as shown in Figure 1. During movement, small colonies started from their inoculation site and became larger when reached to periphery and it followed a translucent path from inoculation site to the periphery. The parental bacterial colony MS1-8 was the mixture of two highly associated bacterial species MS1-8-I and MS1-8-II which were separated and purified later. On the solid surface of the agar plate, the bacterial colonies of MS1-8-I appeared as a white, round shape, small, about 2 mm in diameter, convex, opaque, and smooth margin. Like MS1-8-I, bacterial colonies of MS1-8-II were also in round shape, small, about 2 mm in diameter and smooth margin but it was thin, flat and translucent (Figure 1). Both the bacterial species MS1-8-I and MS1-8-II were Gram-positive. During observation of nutrient broth culture under light microscope, the bacterial species MS-1-8 appeared like small cocci, which was not moving in broth culture while, MS1-8-II appeared as a rod shape and was very fast moving.

The comparative biochemical reactions of bacterial isolates MS1-8-I, MS1-8-II and MS1-8 are summarized in Table 2. All bacterial isolates MS1-8-I, MS1-8-II, and MS1-8 have some common biochemical reactions. They were able to hydrolyze the Esculin and urea. Phenylalanine deamination and Arginine decarboxylation activity were also detected by these three isolates. These

Table 2. Biochemical characteristics of bacterial Isolates MS1-8-I, MS1-8-II and MS1-8.

Test	MS1-8-I (White)	MS1-8-II (Transperant)	MS1-8 (Mixed)
ONPG	+	-	+
PYR	+	-	-
Lysine utilization	+	+	V
Ornithine utilization	+	+	V
Arginine utilization	-	-	-
Urease	+	+	+
Phenylalanine deamination	V	V	V
Nitrate reduction	-	-	+
H_2S production	V	+	+
Citrate utilization	-	+	+
Voges Proskauer's	+	-	-
Methyl Red	-	-	-
Indole	-	-	-
Malonate utilization	-	-	-
Esculin hydrolysis	+	+	+
Arabinose	-	-	+
Xylose	-	-	-
Adonitol	-	-	-
Rhamnose	-	-	-
Cellobiose	-	-	+
Melibiose	-	-	+
Saccharose	+	-	+
Raffinose	-	-	+
Trehalose	+	-	+
Glucose	+	-	+
Arabinose	-	-	+
Sucrose	+	V	+
Sorbitol	-	-	+
Mannitol	+	-	+
Lactose	-	-	+
Oxidase	-	+	+

+, Positive; -, negative; V, variable.

isolates exhibited negative result for utilization of xylose, adonitol, rhamnose, malonate and methyl red. They were not capable of domination of tryptophan, hence showed a negative result for Indole test. The bacterial isolates MS1-8-I and MS1-8 have the ability to utilization of carbohydrates like saccharose, trehalose, glucose, sucrose, and mannitol, while the strain MS1-8-II was not utilizing this carbohydrate as a food sources. Apart from these mentioned carbohydrates, the isolate MS1-8 showed a positive response to utilization of some other carbohydrates like arabinose, cellobiose, melibiose, raffinose, arabinose, sorbitol and lactose. MS1-8 and MS1-8-II also have the capability to use citrate as a carbon source, while MS1-8-I does not have this ability. Only in MS1-8-I, PYR enzyme activities were detected and it also produces the acetoin which confirms positive activity for Voges-Proskauer's reaction. MS1-8-II and MS1-8 have the capability for the production of H_2S and also have the ability for the production of cytochrome oxidase (Table 2).

Matrix-assisted laser desorption / ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis

MALDI-TOF mass spectrum analysis is recently very useful, easy and discriminatory tool for identification of bacterial species (Lista et al., 2011). The obtained score values were compared to Biotyper database for identification of bacterial species. The score value of

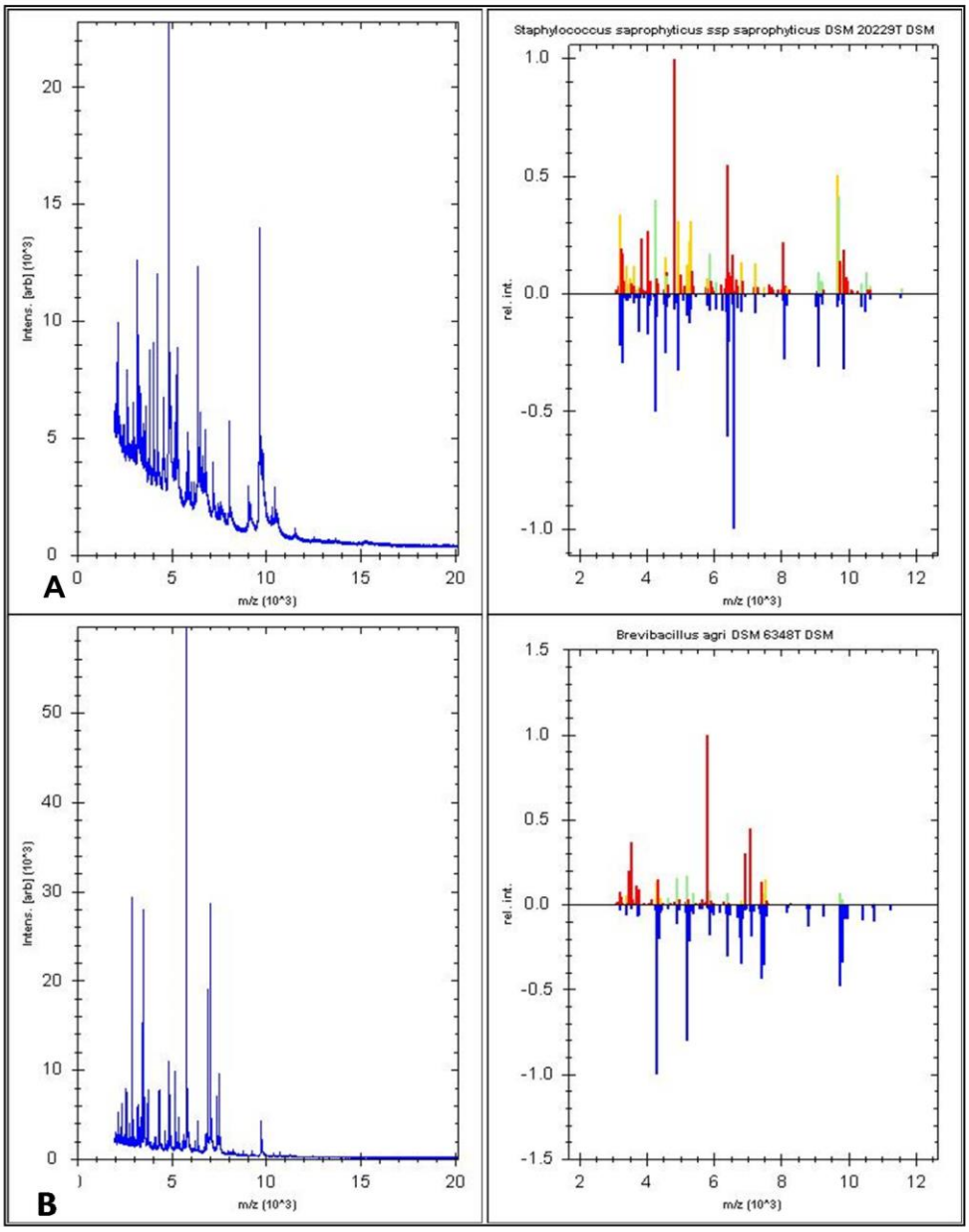

Figure 2. MALDI-TOF mass spectrum analysis based bacterial identification: **A.** *Staphylococcus saprophyticus;* and **B.** *Brevibacillus agri.*

MS1-8-I and MS1-8-II was 1.4 and 1.64 respectively and according to result matching with Biotyper database they were identified as *Staphylococcus saprophyticus* and *Brevibacillus agri* (Figure 2A and B).

16S rRNA gene sequence based analysis

Identification of bacterial species from MALDI-TOF mass spectrum analysis system was finally confirmed with the result of 16S rRNA gene sequence analysis. The obtained sequences of MS1-8-I and MS1-8-II were compared with homologous sequence of Genbank database. The sequence of MS1-8-I showed highest similarity with the sequence of *S. saprophyticus* in EzTaxon server (100%) and NCBI database (100%) and MS1-8-II with *B. agri,* EzTaxon server (99.41%) and NCBI database (100%). The phylogenetic tree was constructed using homologous sequences downloaded from Genbank database in which *Kocuria palustris*

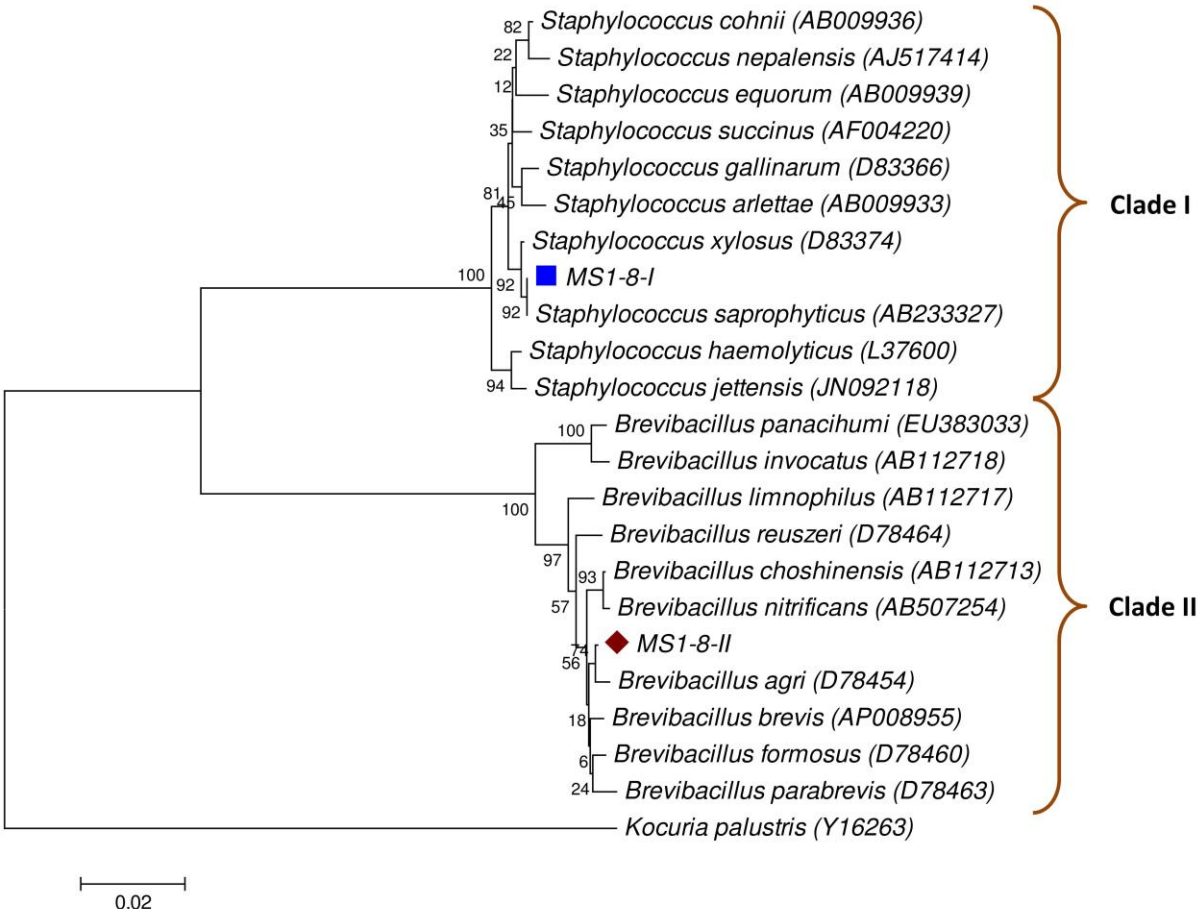

Figure 3. Dendogram based on 16S rRNA gene sequencing constructed by neighbor joining method-using Kimura 2 distances parameter.

(Y16263) was taken as an outgroup. The phylogenetic tree was divided into two mega clusters, genus *Staphylococcus* and *Brevibacillus* (Figure 3).

Scanning Electron Microscopy (SEM)

Bacterial samples were also analyzed through Scanning Electron Microscopy (SEM) at 5000X magnification (Figure 4). The image of MS1-8 containing two types of bacterial cells, one a small cocci and second a long rod shape (Figure 4a). These types of bacterial cells were also appeared in the Gram staining. The bacterial isolate MS1-8-I was identified as *S. saprophyticus* and appeared as a small round shape (Figure 4b) while bacterial isolate MS1-8-II appeared as a long, rod shape and was identified as *B. agri.* The appearence of only two types of bacterial cells in the image of MS1-8, confirmed that the parental bacterial culture MS1-8 was the mixture of two type of bacterial species *S. saprophyticus* and *B. agri* (Figure 4a, b and c).

PCR-RFLP analysis of 16S rRNA gene

According to the examination of broth culture of MS1-8 under a light microscope, Gram staining and SEM image analysis, it was confirmed that the bacterial culture MS1-8 was the mixture of only two different bacterial species and was identified as *S. saprophyticus* (MS1-8-I) and *B. agri* (MS1-8-II), respectively. For further validation of the above finding, we performed the Restriction Fragment Length Polymorphism (RFLP) analysis of 16S rRNA gene of all the bacterial isolates using four different restriction enzymes (MvaI, EcoRI, SphI, and MspI). In the all agarose gel image, the GeneRuler 100 bp Plus DNA Ladder (Thermo fisher scientific, UK) was loaded in the first lane followed by control (uncut 16S rRNA gene), MS1-8-I, MS1-8-II, and MS1-8, respectively. Figure 5A was the gel image digested through enzyme MvaI, where three bands were seen in third lane (MS1-8-I) and two in forth lane (MS1-8-II). All these bands were also seen in the fifth lane (MS1-8). Figure 5B was the image digested by the enzyme EcoRI where two bands appeared in the

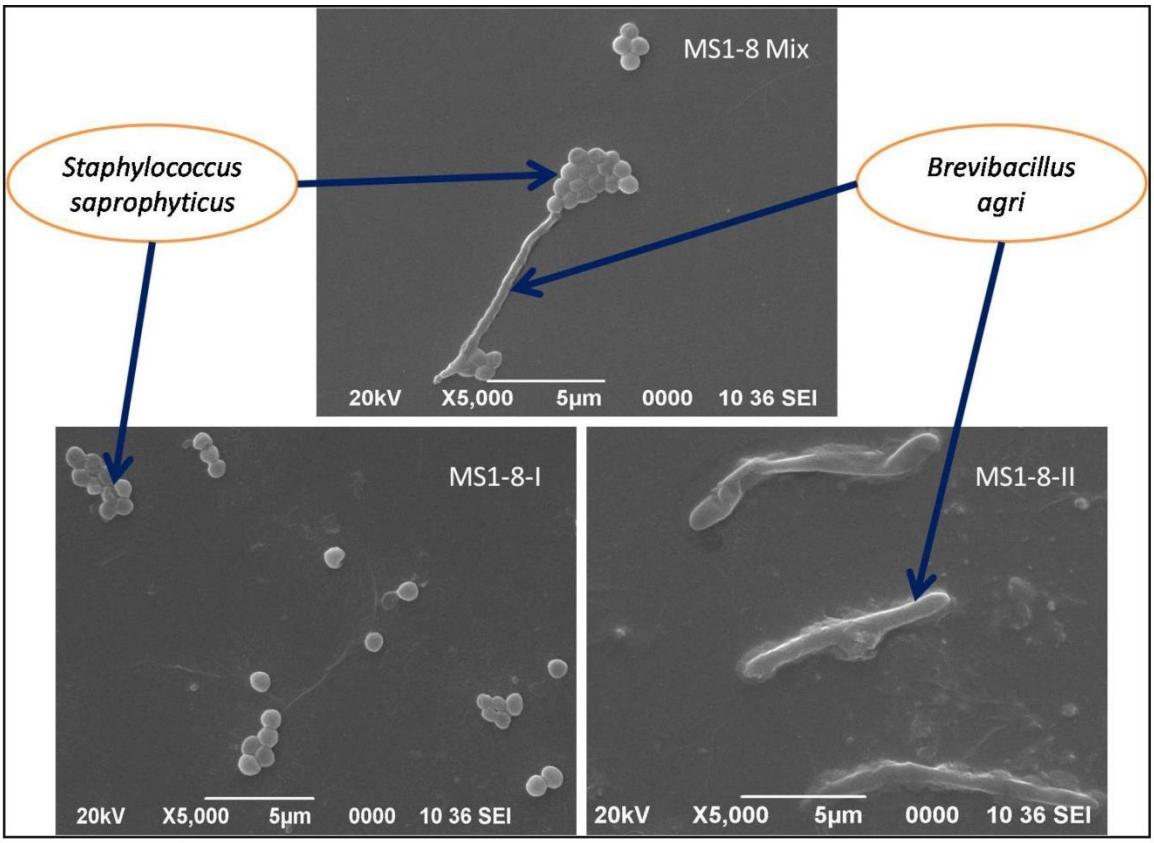

Figure 4. Scanning electron microscopy (SEM) image of bacterial samples at 5000X magnification: (A) Top image MS1-8, (B) below left MS1-8-I (*Staphylococcus saprophyticus*) and (C) below right MS1-8-II (*Brevibacillus agri*).

third lane (MS1-8-I), while in the fourth lane (MS1-8-II), PCR product was uncut and only a single band of 1500 bp size appeared and all these bands were also seen in the fifth lane of sample MS1-8. In Figure 5C, SphI enzyme was used for the digestion but this was unable to cut PCR product, due to which only a single band of about 1500 bp appeared in all lane of MS1-8-I, MS1-8-II, and MS1-8 samples. Last figure 5D was the image digested by the enzyme MspI and the band appeared in third and fourth lane (MS1-8-I and MS1-8-II respectively) was also seen in the fifth lane (MS1-8) (Figure 5).

Movement property study using isolated bacterial species

We have tried to find out the regaining of movement property after mixing of the both pure bacterial species MS1-8-I and MS1-8-II in a specific proportion. The growth and movement properties were observed after 12 to 24 h of incubation and we observed that no any movement properties were regained in the mixed bacterial species MS1-8-I and MS1-8-II, inoculated after 0, 24 and 48 h. It has been also observed that the movement properties were also not regained in the plate where the one bacterial species was inoculated on the lawn of another species.

DISCUSSION

The swimming property of many bacteria in the liquid medium is well studied; however, moving on top of the solid surface of some bacteria is not well characterized. The movement of the bacteria on the solid surface is known as swarming. For the movement of bacteria on a solid surface, various mechanisms, like reduction of surface tension for spreading by secreting some surfactant, increasing flagella, cell-cell interactions and formation of a multicellular group rather than an individual are being employed (Kearns, 2010). In our study, the midgut bacteria isolated from *Ae. albopictus,* when inoculated on center of the nutrient agar plate, exhibited swarming property. These bacteria demonstrated swarming property because they migrated away on the solid surface from their inoculation point (Kearns, 2010). The purified bacterial isolates were initially characterized morphologically and biochemically followed by MALDI-

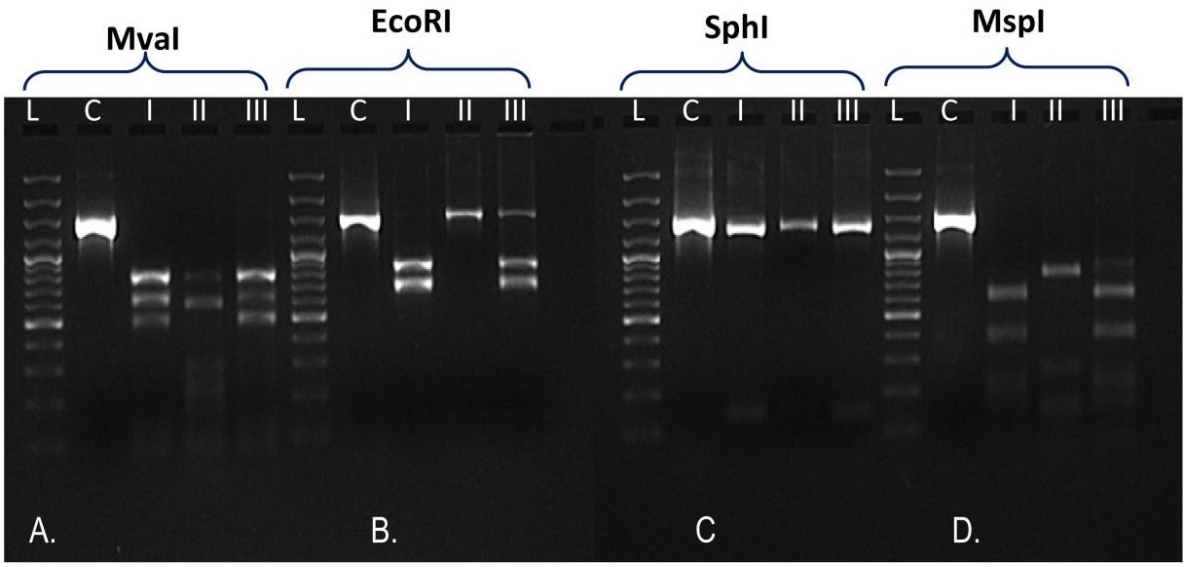

Figure 5. RFLP band pattern of all three bacterial isolates using four different restriction enzymes (MvaI, EcoRI, SphI and MspI). L, ladder; C, control (uncut 16S rRNA gene); I, digested 16S rRNA gene of MS1-8-I; II, digested 16S rRNA gene of MS1-8-II; III, digested 16S rRNA gene of MS1-8.

TOF-MS analysis and 16S rRNA gene sequencing and the result revealed that purified bacterial isolates were *S. saprophyticus* and *B. agri.* The midgut bacteria MS1-8 was the mixture of two bacterial species *S. saprophyticus* and *B. agri*, which was confirmed by the observation of broth culture under microscope, SEM image and RFLP band pattern analysis. Both the bacterial species *S. saprophyticus* and *B. agri* were highly associated during the swarming motility and were not separated easily. Only the bacterial colony MS1-8 showed the swarming property and individually purified bacterial species MS1-8-I and MS1-8-II and the mixture of these purified individuals were not able to show this property. The colony movement property in bacteria MS1-8 might have occurred due to the production of surfactant, which might be secreted by sharing resources and regulated by the quorum sensing property. Sufficient amount of bacteria should be needed for the production of beneficial surfactants, which is required for swarming (Lindum et al., 1988; Ochsner et al., 1995; Eberl et al., 1996; Magnuson et al., 1994). According to our result it is clear that the bacterial isolates MS1-8-I and MS1-8-II were found in very high association in the MS1-8 colony and this highly association-ship of bacterial species might provide the facility for the production of sufficient amount of beneficial surfactants. After the mixing of purified bacterial isolates, this property was not regained, this occurred because they might not be in such a proportion which is required for the production of sufficient amount of surfactant. It has been reported that different bacterial colonies produce different swarming patterns, which depends on the condition of environment where they are growing (Shimada et al., 2004, Hiramatsu et al., 2005).

The bacteria MS1-8 was isolated from midgut of *Ae. albopictus* mosquito where environmental condition of mosquito's midgut was highly different from the environmental condition of the laboratory. Bacterial species residing in the mosquito's midgut were highly associated to each other and fulfilled their requirements for such type of properties till purified two bacterial species in the laboratory conditions. After purification, both bacterial isolates independently were grown on media and lost all connections between them, which was in co-culture MS1-8 (prior to separation). This might be fact that after purification swarming property was not regained in individuals or in the mixture of the two purified bacterial species.

Apart from a surfactant, cell-cell interactions, and flagella formation is also an important requirement for swarming property. In our study, in the broth culture of purified bacterial species, *Staphylococcus saprophyticus* is Gram positive, cocci, and non-motile while the *B. agri* is Gram positive, rod shape, and highly motile. It has been reported that bacteria *S. saprophyticus* do not have flagella while in *B. agri,* it is present (Lara-Mayorga et al., 2010; Joshi et al., 2013). The presence of flagella in *B. agri* might play an important role in swarming property of parental isolated co-culture MS1-8, which might be supported by *S. saprophyticus.*

A lot of bacteria have been isolated from various mosquito's midgut and its role in various important functions like; food digestion, pathogen growth inhibition

etc. have been also studied (Minard et al., 2013). The identified bacterial species *S. saprophyticus* and *B. agri* in our studies have already previously reported from different mosquito's midgut. The genera *Staphylococcus* was normally isolated from the midgut of *Culex, Anopheles* and *Aedes* mosquitoes, while *Brevibacillus* was scarcely isolated (Chandel et al., 2013; Moro et al., 2013; Ramirez et al., 2012; Cirimotich et al., 2011a; Zouache et al., 2011; Rani et al., 2009; Favia et al., 2007; Fouda et al., 2001; Pumpuni et al., 1993; Apte-Deshpande et al., 2012; Ngwa et al., 2013). Different essential functions of midgut microorganisms in various fields have been depicted but swarming properties were not previously reported.

It has been previously described that the midgut bacteria provides viable defense against invading pathogens. The midgut bacteria are responsible for the inhibition of sporogonic development (Pumpuni et al., 1993; Straif et al., 1998; Gonzalez-Ceron et al., 2003; Dong et al., 2006; Cirimotich et al., 2011b). The bacterial species interact with host-pathogens and bacterial species itself and plays important role in vectorial capacity of mosquitoes. The bacterial species inside the midgut interact with each other, but the information about these interactions, its behavior is limited. Using midgut bacteria in vector-borne disease control, it is important to find out more information regarding bacterial species interaction. In this study, we have characterized the bacterial species responsible for the swarming motility, which was an example of bacterial interaction. The information obtained from this study might be a step towards the understanding of midgut bacterial interaction, which will help in development of a novel, cheap and eco-friendly approach towards the prevention of any vector-borne disease.

Conflict of Interests

The authors declare that they do not have any conflict of interest.

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REFERENCES

Alam SI, Dixit A, Reddy GS, Dube S, Palit M, Shivaji S, (2006). *Clostridium schirmacherense* sp. nov., an obligately anaerobic, proteolytic, psychrophilic bacterium isolated from lake sediment of Schirmacher Oasis, Antarctica. Int. J. Syst. Evol. Microbiol. 56:715-720.

Allison C, Emody L, Coleman N, Hughes C (1994). The role of swarm cell differentiation and multicellular migration in the uropathogenicity of *Proteus mirabilis*. J. Infect. Dis. 169:1155-1158.

Apte-Deshpande A, Paingankar M, Gokhale MD, Deobagkar DN (2012). *Serratia odorifera* a midgut inhabitant of *Aedes aegypti* mosquito enhances its susceptibility to dengue-2 Virus. PLoS One *7*:e40401.

Apte-Deshpande AD, Paingankar MS, Gokhale MD, Deobagkar DN (2014). *Serratia odorifera* mediated enhancement in susceptibility of *Aedes aegypti* for chikungunya virus. Indian J. Med. Res. 139(5):762.

Boissière A, Tchioffo MT, Bachar D, Abate L, Marie A, Nsango SE, Shahbazkia HR, Awono-Ambene PH, Levashina EA, Christen R, Morlais I (2012). Midgut microbiota of the malaria mosquito vector *Anopheles gambiae* and interactions with *Plasmodium falciparum* infection. PLoS Pathog. 8:e1002742.

Chandel K, Mendki MJ, Parikh RY, Kulkarni G, Tikar SN, Sukumaran D, Prakash S, Parashar BD, Shouche YS, Veer V (2013). Midgut microbial community of *Culex quinquefasciatus* mosquito populations from India. PLoS ONE 8(11):e80453. doi: 10.1371/journal.pone.0080453.

Cirimotich CM, Clayton AM, Dimopoulos G (2011a). Low- and high-tech approaches to control *Plasmodium* parasite transmission by *Anopheles* mosquitoes. J. Trop. Med. 891342.

Cirimotich CM, Dong Y, Clayton AM, Sandiford SL, Souza-Neto JA, Mulenga M, Dimopoulos G (2011b). Natural microbe-mediated refractoriness to *Plasmodium* infection in *Anopheles gambiae*. Science 332(6031):855-858.

Cong Y, Wang J, Chen Z, Xiong K, Xu Q, Hu F (2011). Characterization of swarming motility in *Citrobacter freundii*. FEMS Microbiol. Lett. 317(2):160-171.

Dillon R, Charnley K (2002). Mutualism between the desert locust *Schistocerca gregaria* and its gut microbiota. Res. Microbiol. 153:503-509.

Dong Y, Aguilar R, Xi Z, Warr E, Mongin E, Dimopoulos G (2006). *Anopheles gambiae* immune responses to human and rodent *Plasmodium* parasite species. PLoS Pathog. 2:e52.

Dong Y, Manfredini F, Dimopoulos G (2009). Implication of the Mosquito Midgut Microbiota in the Defense against Malaria Parasites. PLoS Pathog 5(5):e1000423.

Eberl L, Winson MK, Sternberg C, Stewart GSAB, Christiansen G, Chhabra SR, Bycroft B, Williams P, Molin S, Givskov M (1996). Involvement of N-acyl-L-homoserine lactone autoinducers in controlling the multicellular behavior of *Serratia liquefaciens*. Mol. Microbiol. 20:127–136.

Favia G, Ricci I, Damiani C, Raddadi N, Crotti E, Marzorati M, et al. (2007). Bacteria of the genus *Asaia* stably associate with *Anopheles stephensi*, an Asian malarial mosquito vector. Proc. Natl. Acad. Sci. USA 104: 9047–51.

Fouda MA, Hassan MI, Al-Daly AG, Hammad KM (2001). Effect of midgut bacteria of *Culex pipiens* L. on digestion and reproduction. J. Egypt. Soc. Parasitol. 31:767–780.

Fraser GM, Hughes C (1999). Swarming motility. Curr. Opin. Microbiol. 2:630–635.

Gonzalez-Ceron L, Santillan F, Rodriguez MH, Mendez D, Hernandez-Avila JE (2003). Bacteria in midguts of field-collected *Anopheles albimanus* block *plasmodium vivax* sporogonic development. J. Med. Entomol. 40:371–374.

Harshey RM (2003). Bacterial motility on a surface: Many ways to a common goal. Annu. Rev. Microbiol. 57:249–273.

Hiramatsu F, Wakita J-I, Kobayashi N, Yamakazi Y, Matsushita M, Matsuyama T (2005). Patterns of expansion produced by a structured cell population of *Serratia marscescens* in response to different media. Microbes Environ. 20:120–125.

Joshi MN, Sharma A, Pandit AS, Pandya RV, Saxena AK, Bagatharia

SB (2013). Draft Genome Sequence of *Brevibacillus* sp. Strain BAB-2500, a Strain That Might Play an Important Role in Agriculture. Genome Announc 1(1). pii: e00021-13.
Kaiser D (2007). Bacterial swarming: A re-examination of cell movement patterns. Curr. Biol. 17:561–570.
Kearns DB (2010). A field guide to bacterial swarming motility. Nat. Rev. Microbiol. 8(9): 634–644. doi:10.1038/nrmicro2405.
Kim W, Killam T, Sood V, Surette MG (2003). Swarm-cell differentiation in Salmonella enterica serovar Typhimurium results in elevated resistance to multiple antibiotics. J. Bacteriol. 185:3111–3117.
Lara-Mayorga I, Durán-Hinojosa U, Arana-Cuenca A, Monroy-Hermosillo O, Ramírez-Vives F (2010). Vinyl acetate degradation by *Brevibacillus agri* isolated from a slightly aerated methanogenic reactor. Environ. Technol. 31(1):1-6.
Lindum PW, Anthoni U, Christophersen C, Eberl L, Molin S, Givskov M (1988). N-acyl-L-homoserine lactone autoinducers control production of an extracellular lipopeptide biosurfactant required for swarming motility in *Serratia* liquefaciens MG1. J. Bacteriol. 180:6384-6388.
Lista F, Reubsaet FA, De Santis R, Parchen RR, de Jong AL, Kieboom J, van der Laaken AL, Voskamp-Visser IA, Fillo S, Jansen HJ, Van der Plas J, Paauw A (2011). Reliable identification at the species level of *Brucella* isolates with MALDI-TOF-MS. BMC Microbiol. 11(1):267.
Magnuson R, Solomon J, Grossman AD (1994). Biochemical and genetic characterization of a competence pheromone from *B. subtilis*. Cell 77:207-216.
Merino S, Shaw JG, Tom´as JM (2006). Bacterial lateral flagella: an inducible flagella system. FEMS Microbiol. Lett. 263:127-135.
Minard G, Mavingui P, Moro CV (2013). Diversity and function of bacterial microbiota in the mosquito holobiont. Parasit. Vectors. 6:146.
Moro CV, Tran FH, Raharimalala FN, Ravelonandro P, Mavingui P (2013). Diversity of culturable bacteria including *Pantoea* in wild mosquito *Aedes albopictus*. BMC Microbiology 13:70.
Ngwa CJ, Ckner VG, Abdelmohsen UR, Scheuermayer M, Fischer R, Hentschel U, Pradel G (2013). 16S rRNA gene-based identification of *Elizabethkingia meningoseptica* (Flavobacteriales: Flavobacteriaceae) as a dominant midgut bacterium of the Asian malaria vector *Anopheles stephensi* (Dipteria: Culicidae) with antimicrobial activities. J. Med. Entomol. 50(2):404-414.
Ochsner UA, Reiser J (1995). Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA 92:6424-6428.
Prakash A, Thavaselvam D, Kumar A, Kumar A, Arora S, Tiwari S, Barua A, Sathyaseelan K (2014). Isolation, identification and characterization of *Burkholderia pseudomallei* from soil of coastal region of India. Springer Plus 3:438.
Pumpuni CB, Beier MS, Nataro JP, Guers LD, Davis JR (1993). *Plasmodium falciparum*: inhibition of sporogonic development in *Anopheles stephensi* by Gram-negative bacteria. Exp. Parasitol. 77:195-199.
Ramirez JL, Souza-Neto J, Cosme RT, Rovira J, Ortiz A, Juan M. Pascale JM, Dimopoulos G (2012). Reciprocal Tripartite Interactions between the *Aedes aegypti* Midgut Microbiota, Innate Immune System and Dengue Virus Influences Vector Competence. PLoS Negl. Trop. Dis. 6(3):e1561.
Rani A, Sharma A, Rajagopal R, Adak T, Bhatnagar RK (2009). Bacterial diversity analysis of larvae and adult midgut microflora using culture-dependent and culture-independent methods in lab-reared and field-collected *Anopheles stephensi*- An Asian malarial vector. BMC Microbiol. 9:96.
Rueda LM (2004). Picrorial keys for identification of mosquitoes (Dipter: Culicidae) associated with Dengue Virus Transmission. Magnolia Press, Auckland, New Zealand.
Sambrook J, Russell DW (2001). Molecular Cloning: A Laboratory Manual; 3, editor: Cold Spring Harbor Laboratory Press.
Shimada H, Ikeda T, Wakita J-I, Itoh H, Kurosu H, Hiramatsu F, Nakatsuchi M, Yamazaki Y, Matsuyama T, Matsushita M (2004). Dependence of local cell density on concentric ring colony formation by bacterial species *Bacillus subtilis*. J. Phys. Soc. Japan 73:1082-1089.
Stickler D, Morris N, Moreno MC, Sabbuba N (1998). Studies on the formation of crystalline bacterial biofilms on urethral catheters. Eur. J. Clin. Microbiol. 17:649-652.
Straif SC, Mbogo CN, Toure AM, Walker ED, Kaufman M, Toure YT, Beier JC (1998). Midgut bacteria in *Anopheles gambiae* and *An. funestus* (Diptera: Culicidae) from Kenya and Mali. J. Med. Entomol. 35:222-226.
Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol. Biol. Evol. 30(12):2725-2729.
Terenius O, Lindh JM, Eriksson-Gonzales K, Bussière L, Laugen AT, Bergquist H, Titanji K, Faye I (2012). Midgut bacterial dynamics in *Aedes aegypti*. FEMS Microbiol. Ecol. 80:556-565.
Yadav KK, Bora A, Datta S, Chandel K, Gogoi HK, Prasad GB, Veer V (2015a). Molecular characterization of midgut microbiota of *Aedes albopictus* and *Aedes aegypti* from Arunachal Pradesh, India. Parasit. Vectors 188(1):641.
Yadav KK, Chandel K, Bora A, Veer V (2015b). Isolation and characterization of *Dietzia maris* from midgut of *Aedes albopictus*: A suitable candidate for paratransgenesis. Int. J. Mosq. Res. 7(24):7-12.
Zouache K, Michelland RJ, Failloux A-B, Grundmann GL, Mavingui P (2012). Chikungunya virus impacts the diversity of symbiotic bacteria in mosquito vector. Mol. Ecol. 21:2297-2309.
Zouache K, Raharimalala FN, Raquin V, Tran-Van V, Raveloson LH, Ravelonandro P, Mavingui P (2011). Bacterial diversity of field-caught mosquitoes, *Aedes albopictus* and *Aedes aegypti*, from different geographic regions of Madagascar. FEMS Microbiol. Ecol. 75:377-389.


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