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INACTIVATION OF SHIGA-TOXIN PRODUCING *Escherichia coli* (STEC) O157:H7 IN MILK BY COMBINED TREATMENT WITH HIGH HYDROSTATIC PRESSURE AND AQUEOUS POMEGRANATE EXTRACT

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ABSTRACT

The aim of this work was to evaluate the synergistic effect of combined treatment with high hydrostatic pressure (HHP) and an aqueous extract of *Punica granatum* (pomegranate) peels on the survival of Shiga toxin-producing *Escherichia coli* (STEC). Our results showed that HHP (250 MPa, 60 min, 25 ºC) reduced the STEC bacterial load in milk and tryptic soy broth culture medium by about 2.5 and 3.5 logs, respectively. Under these conditions, HHP did not alter the cytotoxicity of Shiga toxins in Vero and human umbilical vein endothelial cells. Treatment with up to 30 mg of pomegranate extract/mL caused negligible inactivation, but a combination of HHP and pomegranate extract (3 mg/mL) produced bacterial inactivation from 10^9 CFU/mL to undetectable levels of viable bacteria. These findings suggest that a combination of HHP and pomegranate extract may be potentially effective in bacterial inactivation during food processing, particularly in the elimination of important foodborne pathogens such as STEC. a

Keywords: *Escherichia coli*, High Hydrostatic Pressure, Pomegranate extract, Shiga toxins, STEC

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) are important food and waterborne bacterial pathogens. These microorganisms produce potent cytotoxins, called Shiga toxins (Stx) that, for more than three decades, have been linked to large outbreaks of gastrointestinal illness **(Manning *et al*., 2008)**. STEC strains, particularly *E. coli* O157:H7, cause a wide range of illnesses such as bloody diarrhea, hemorrhagic colitis, hemolytic uremic syndrome (HUS) and, in some cases, death **(Kaper *et al*., 2004)**.

Many cases of foodborne diseases worldwide are associated with the consumption of STEC-contaminated ground beef, water, raw milk, unpasteurized fruit juices, vegetables and other fresh produce **(Yoon and Hovde, 2008; FDA, 2012)**. Contamination with as few as ten STEC O157:H7 strain bacteria is sufficient to cause human infection **(FDA, 2009)**. Recent studies estimate that in the United States of America the mean annual cost of treating foodborne illnesses caused by STEC O157:H7 strains is ~US$ 635 million **(Scharff, 2012)**.

Cattle are the major reservoir for STEC and undercooked ground beef is one of the most common causes of STEC O157:H7 strain infections **(Riley *et al*., 1983; Rangel *et al*., 2005)**. The consumption of contaminated raw milk dairy products may also be an important route of STEC infection **(Miszczycha *et al*., 2014)**. Contamination rates of up to 10% have been reported for STEC O157:H7 in bulk tank milk samples collected in North America **(D'Amico *et al*., 2010)**.

Pasteurization is the recommended procedure for controlling the microbiological risks associated with the consumption of raw milk since it substantially decreases or eliminates pathogens and effectively prevents disease transmission **(Guh *et al*., 2010)**. However, high temperatures may cause detrimental changes to processed foods, leading to undesirable changes that can affect the nutritional and organoleptic characteristics, resulting in products that are nutritionally different when compared to similar fresh products **(San Martín *et al*., 2002)**.

Non-thermal techniques, including high hydrostatic pressure (HHP), have received considerable interest from the food industry, with pressures of 100-1200 MPa being particularly effective in the inactivation of a wide variety of microorganisms, including foodborne pathogens. Another important attribute of HHP is that this technique also improves rennet or acid coagulation of milk with no detrimental effect on nutritional quality, flavor and texture **(Chawla *et al*., 2011)**.

HHP is a powerful tool for developing novel dairy products of better nutritional and sensory quality, novel texture and increased shelf-life. Non-thermal processing may improve the energy efficiency of food processing. For these reasons, high pressure technology is increasingly being used to produce value-added food products. HHP can be used to process liquid and solid (water-containing) foods, with the advantages that it kills bacteria in raw food, extends the shelf-life and enhances desired attributes, e.g., digestibility **(Makhal *et al*., 2003)**.

Appropriate combinations of pressure and temperature may be a useful alternative to achieve a suitable synergistic effect in reducing the bacterial load in foods **(Moussa *et al*., 2006)**. The antibacterial activity of aqueous and ethanolic extracts of *Punica granatum* (pomegranate) peels against different pathogens, such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*, has been described **(Reddy *et al*., 2007)**. The exceptional antioxidant potential and promising medicinal properties of pomegranate peels have led to intensive research of its role in human health **(Lansky and Newman, 2007)**. An extract of pomegranate peels has been shown to inhibit the growth of STEC O157:H7 strain **(Howell and D'Souza, 2013)** and also exhibited antidiarrheal properties. The presence of water-soluble polyphenols in the aqueous extract of pomegranate peels may mediate its antidiarrheal activity **(Qnais *et al*., 2007)**.

Based on the foregoing studies, the objective of this work was to evaluate the effect of HHP (250 MPa for 10, 20, 30, 45 and 60 min at 25 °C) combined with different concentrations of an aqueous extract of pomegranate on the survival of STEC O157:H7 inoculated into different substrates (milk and culture medium). The effect of HHP (250 MPa, 60 min, 25 °C) on the biological activity of the Shiga toxins (Stx1 and Stx2) produced by STEC O157:H7 strain was also evaluated. Understanding the behavior of STEC strains under different synergistic conditions may be useful for developing more effective methods of inactivating foodborne pathogens.

MATERIAL AND METHODS

Strains

The following *E. coli* strains were used for the different assays: O157:H7 EDL933 (produces Stx1 and Stx2), C600 K-12 (does not produce Stx1 and Stx2),

C600 H19B (produces only Stx1) and C600 933W (produces only Stx2). All of the strains were from the bacterial collection of the Laboratory of Virulence Factors of the Institute of Biology at UNICAMP (Campinas, SP, Brazil).

HHP system

HHP was generated by a pump operated by an engine that injected ethanol via a pipeline (HIP model 37-5.75-60; Erie, PA, USA) into a metal chamber (external dimensions, 91.3 mm wide x 91.3 mm deep; internal volume capacity, 24.5 mL; nominal high pressure capacity up to 400 MPa; ISS HP Cell, ISS Inc., Champaign, IL, USA) **(Bispo *et al*., 2007)**. The time required to increase the pressure from atmospheric pressure to 250 MPa was 2.5 min and the decompression time was 1.0 min. The pressure chamber was connected to a circulating water bath fitted with a temperature controller (Marconi, Piracicaba, SP, Brazil). For HHP treatment, the different samples were placed in polyethylene bags, sealed at high temperature and placed in the high pressure chamber **(Souza *et al*., 2013)**.

Preparation of milk contaminated with *E. coli* O157:H7 strain

Contaminated milk was prepared as described by **Durães-Carvalho *et al*. (2012)**, with slight modifications. *E. coli* O157:H7 EDL933 strain was cultured in tryptic soy broth (TSB) and incubated at 37 °C overnight. The number of bacteria was adjusted to ~10^9 CFU/mL using the McFarland nephelometric scale. The bacterial suspension was centrifuged at 10,000 × *g* and the supernatant was discarded. The bacterial pellet was washed three times with sterile PBS, pH 7.2, and resuspended in filter-sterilized whole milk for immediate use in the HHP assays.

Preparation of aqueous pomegranate extract

Pomegranate fruits were washed with distilled water and air-dried. Pomegranate peel was removed and 40 g was placed in an Erlenmeyer flask. After adding 100 mL of distilled water, the mixture was stored in the dark at 25 °C for 24 h and sterilized by filtration using 0.22 µm pore-sized filters (Millipore, São Paulo, SP, Brazil). The extract was lyophilized and resuspended in sterile distilled water, filter-sterilized and stored at -20 °C until use. The pomegranate species was identified at the Department of Plant Biology, Institute of Biology at UNICAMP), Brazil, and a voucher specimen (code UEC037017) was deposited in the Herbarium of this Department.

HHP assays

Inactivation of E. coli O157:H7 strain by HHP

E. coli O157:H7 EDL933 was cultured in TSB and incubated overnight at 37 °C with shaking. The bacterial cultures were adjusted to a cell concentration of ~10^9 CFU/mL using the McFarland nephelometric scale. The bacterial suspension and samples of whole milk contaminated with *E. coli* O157:H7 EDL933 were used for the pressure treatment assays. A pressure of 250 MPa was applied to each sample at 25 °C for various times (10, 20, 30, 45 and 60 min) after which the bacterial viability was analyzed by plating 10-fold serial dilutions of bacterial suspensions, prepared in sterile PBS, pH 7.2, on MacConkey agar plates. The plates were incubated at 37 °C for 24 h before counting the colonies. This assay was done in triplicate.

Effect of HHP combined with pomegranate extract

This assay was done under three sets of conditions. Initially, we examined the effect of high pressure (250 MPa, 60 min, 25 °C) on bacterial suspensions in TSB and milk contaminated with *E. coli* O157:H7 EDL933. Next, the antimicrobial effect of a pomegranate aqueous extract was tested. For this, bacterial suspensions in TSB or contaminated whole milk were treated with the pomegranate aqueous extract (3 mg/mL and 30 mg/mL) after which the samples were left at room temperature for 1 h. Finally, the effect of HHP combined with pomegranate extract was examined using the same pressure and time parameters as for the previous two steps. For all three steps, viable bacteria were quantified after incubation at 37 °C for 24 h in MacConkey agar plates.

***In vitro* cytotoxicity assays**

Preparation of cell-free filtrates

E. coli O157:H7 EDL933, C600 H19B, C600 933W and C600 K-12 strains were cultured in TSB and incubated overnight at 37 °C with shaking. The bacterial cultures were centrifuged at 10,000 × *g* for 10 min and the supernatant was sterilized by filtration using 0.22 µm pore-sized membranes before doing the experiments.

MTT reduction assay

The cytotoxicity of the different cell-free filtrates was assessed using the MTT (3-[4,5-dimethylthiazole]-2,5-diphenyltetrazolium bromide thiazole blue) cytotoxicity assay in African green monkey kidney cells (Vero) and human umbilical vein endothelial (HUVEC) cells, according to **Bernedo-Navarro *et al.* (2014)**. One hundred microliters of cell-free filtrate was added to cell-containing wells and the microplates were incubated at 37 °C for 45 h in a 5% CO_2 atmosphere. After this step, the culture medium of each well was discarded and 100 µL of MTT (2 mg/mL in PBS) was added to each well, after which the plate was incubated for 3 h at the same temperature in a 5% CO_2 atmosphere. Finally, the MTT solution was discarded and 100 µL of isopropanol-hydrochloric acid (1 N) (24:1, v/v) was added to each well. The resulting absorbance was read at 450 nm using an ELISA microplate reader (Epoch, BioTek Instruments Inc., VT, USA) and the percentage of viable cells was calculated. This assay was done in triplicate.

Effect of HHP on the cytotoxicity of shiga toxins

To assess whether HHP affected the cytotoxicity of Shiga toxins (Stx), bacterial cultures of *E. coli* O157:H7 (Stx1+ and Stx2+), *E. coli* C600 H19B (Stx1+), *E. coli* C600 (Stx2+) and *E. coli* C600 K-12 (non-producer of Stx) strains were cultured in TSB at 37 °C overnight. After incubation, 1 mL of each bacterial culture was pressurized at 250 MPa for 1 h at 25 °C followed by centrifugation (10,000 × *g*, 10 min, 4 °C). The bacterial supernatant was filter-sterilized and 100 µL of two-fold dilutions of this free-cell filtrate (in DMEM) were assayed for cytotoxicity on Vero and HUVEC cells. Cytotoxicity was monitored using an inverted microscope (Nikon Instruments, Tokyo, Japan) after incubation for 24 h at 37 °C in a 5% CO_2 atmosphere. The results were expressed as the highest dilution at which cytotoxicity was observed (cytotoxicity titer). This assay was done in triplicate.

Statistical analysis

The results were expressed as the mean ± SD (standard deviation). Statistical comparisons were done using GraphPad Prism v.5.01 (GraphPad Software, San Diego, CA, USA; http://www.graphpad.com).

RESULTS

HHP and the viability of *E. coli* in TSB medium and milk

The Fig 1 shows the effect of HHP on the inactivation of *E. coli* O157:H7 EDL933 strain in TSB and milk. Bacterial inactivation was assessed after exposure to high pressure (250 MPa) for different times. The inactivation in TSB (from 10^9 to$10^{5.5}$ log CFU/mL, approximately) was slightly higher than that in contaminated milk (from $10^{8.5}$ to $10^{6.0}$ log CFU/mL).

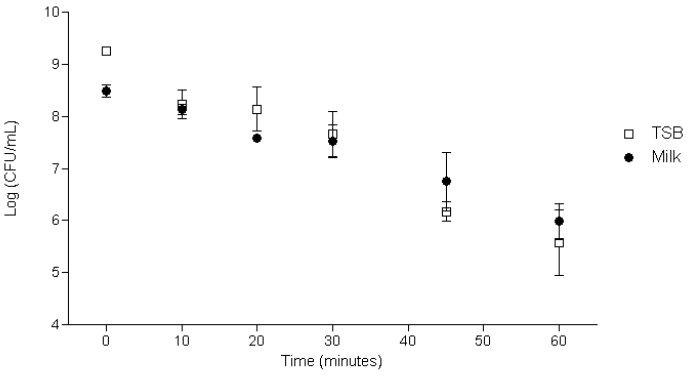

Figure 1 Kinetics for the inactivation of *E. coli* O157:H7 EDL933 by HHP (250 MPa) in TSB medium and milk. The points represent the mean ± SD.

Effect of HHP combined with pomegranate aqueous extract on the viability of *E. coli* O157:H7

EDL933 strain in TSB and whole milk

The Fig 2 shows that treatment of the bacterial culture in TSB medium and milk at 250 MPa for 1 h reduced the bacterial load by 4.0 and 2.5 orders of magnitude, respectively, in agreement with the results in Fig 1. The combination of HHP with pomegranate aqueous extract in TSB and milk contaminated with this pathogen resulted in total inactivation of the bacterial load (from 10^9 to 0 CFU/mL) (Fig 2).

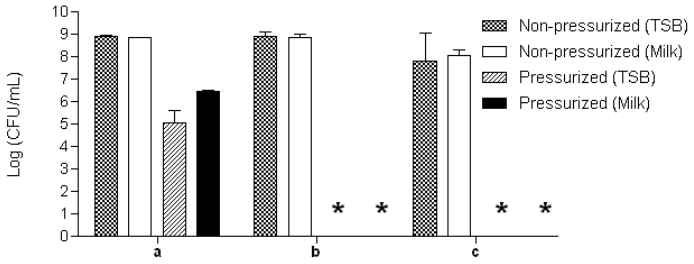

Figure 2 Effect of a combination of HHP (250 MPa, 60 min) and pomegranate aqueous extract on *E. coli* O157:H7 EDL933 inactivation in TSB culture medium and milk. a: No extract; b: 3 mg of extract/mL; c: 30 mg of extract/mL. * No viable bacteria detected. The columns represent the mean ± SD.

Cytotoxicity of pomegranate aqueous extract in VERO and HUVEC cells

At concentrations of 3 mg/mL and 30 mg/mL the pomegranate aqueous extract was cytotoxic to Vero cells and reduced the cell viability by ~50% (Fig 3A), but was not cytotoxic to HUVEC cells (Fig 3B).

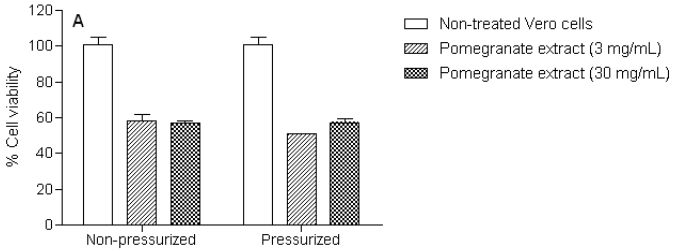

Figure 3 Cytotoxicity of pomegranate aqueous extract in Vero (A) and HUVEC cells (B). The columns represent the mean ± SD.

Cytotoxicity assessed by the MTT assay

Cytotoxicity of E. coli O157:H7 EDL933 strain cell-free filtrates in VERO and HUVEC cells

The cytotoxicity of *E. coli* cultured in TSB medium and subjected to 250 MPa for 1 h was similar in Vero and HUVEC cells (Fig 4). There were no significant differences in cell viability among the different conditions tested (cell-free filtrate, pressurized O157:H7 bacterial culture). HHP did not affect the biological activity of the toxin (Table 1).

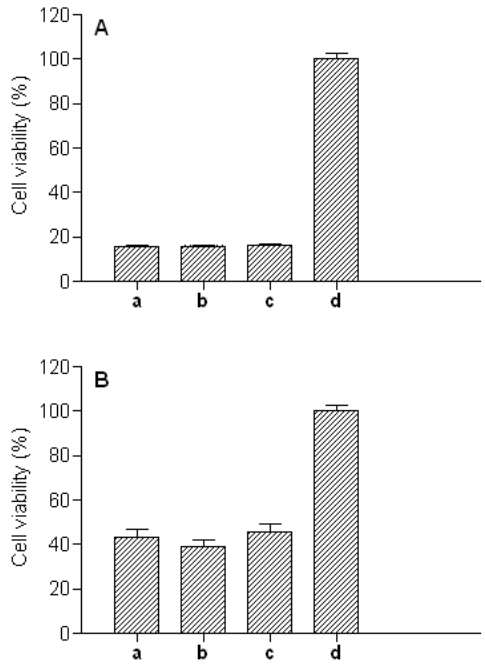

Figure 4 Cytotoxicity of *E. coli* O157:H7 EDL933 cell-free filtrates in Vero (A) and HUVEC cells (B) as assessed by the MTT assay. a: STEC O157:H7 EDL933 cell-free filtrate, b: Pressurized STEC O157:H7 EDL933 cell-free filtrate, c: Pressurized STEC O157:H7 EDL933 culture, d: Non-treated cells. The columns represent the mean ± SD.

Effect of HHP on the cytotoxicity of shiga toxins from different *E. coli* strains

Table 1 shows the effect of HHP on the cytotoxicity of different *E. coli* strains. Exposure of cell-free filtrates of *E. coli* O157:H7 EDL933, *E. coli* C600 H19B and *E. coli* C600 933W strains at 250 MPa for 1 h did not affect the biological activity of Stx1 and Stx2 when compared with non-pressurized samples.

Table 1 Effect of HHP treatment on the cytotoxicity of cell-free filtrates of *E. coli* strains.

E. coli strains	Genotype		Treatment	Cytotoxicity titer in Vero cells
	*stx*1	*stx*2		
C600 K12	-	-	P*	0
			NP	0
O157:H7 EDL933	+	+	P*	1/1024
			NP	1/1024
C600 H19B	+	-	P*	1/256
			NP	1/256
C600 933W	-	+	P*	1/256
			NP	1/256

*P = pressurized (250 MPa, 60 min, 25 °C); NP = non-pressurized

DISCUSSION

The use of HHP is a promising approach for the inactivation of microorganisms in milk and derived products. Despite a low initial bacterial load of STEC O157:H7 in milk, this pathogen can grow during cheese processing to reach levels high enough to be considered a threat to human health **(Montet *et al*., 2009)**. In this context, the use of methods capable of bacterial inactivation may represent an alternative for controlling the levels of STEC strains in milk destined for the manufacturing of products for human consumption **(Farrokh *et al*., 2013)**.

Another promising alternative for reducing bacterial load is the use of pomegranate extract, as has been applied to several bacterial species **(Al Laham and Al Fader, 2013)**. Specific compounds obtained from pomegranate extract, such as punicalagin, when used in combination with classic antimicrobials, can potentiate their effects and significantly reduce the toxic side effects **(Endo *et al*., 2010)**. **Rodriguez *et al*. (2005)** found that pressurization of cheese (300 MPa, 10 min, 10 °C) reduced the bacterial load of STEC O157:H7 to undetectable levels when combined with the application of several bacteriocins. **De Lamo-Castellví *et al*. (2006)** also reported that HHP (400-500 MPa) reduced the bacterial load of STEC O157:H7 in cheese curd.

In the present study, the kinetics of *E. coli* O157:H7 EDL933 inactivation by HHP was examined (Fig 1). The pressure-induced inactivation of STEC O157:H7 EDL933 in TSB culture medium and milk was similar, with a reduction in the bacterial load of ~3 orders of magnitude (from 10^9 to 10^6 CFU/mL). Similar results were observed using 250 MPa for the inactivation of *A. hydrophila* AH 191 strain in whole milk **(Durães-Carvalho *et al*., 2012)**. To improve the HHP-mediated inactivation of *E. coli,* the treatment was combined with aqueous pomegranate extract (3 mg/mL and 30 mg/mL) that is well known to have antimicrobial properties. Total bacterial inactivation was observed with this combination (>10^9 fold reduction) when compared to the extract alone (Fig 2), indicating synergism between HHP and the extract. The TBS or milk in which the bacteria were grown apparently did not influence the inactivation by HHP and extract (Fig 2).

To analyze the cytotoxicity of the pomegranate extract, Vero and HUVEC cells were treated with the aqueous extract (3 and 30 mg/mL). We observed ~50% cell mortality in Vero cells (Fig 3A), while in HUVEC cells no significant cytotoxic effects were observed (Fig 3B). HHP-treated extract and untreated extract showed similar cytotoxicity at both concentrations (Fig 3A,B). According to **Endo *et al*. (2010)**, Vero cells were sensitive to punicalagin, a compound present in large quantities in pomegranate, after application of the extract at a concentration of 60 mg/mL.

Stx-mediated endothelial cell damage may result in bleeding characteristic of hemorrhagic colitis associated with some STEC infections, thereby allowing the toxin to reach the bloodstream and damage endothelial cells at distant sites such as the kidney and brain **(Jacewicz *et al*., 1999)**. However, at the two concentrations tested, the extract had little effect on the viability of HUVEC cells (Fig 3B). This finding suggests that pomegranate extract does not contribute to endothelial cell damage and would not enhance the cytotoxicity of Stx toxins in these cells.

We also examined the effect of HHP on Stx virulence since high pressure inactivates a variety of bacteria and viruses. HHP had little or no effect on the cytotoxicity of these toxins in Vero and HUVEC cells (Fig 4A and Fig 4B, respectively), specifically Shiga toxins 1 and 2 (Stx1 and Stx2) (Table 1). This finding agreed with previous reports showing a lack of bacterial toxin inactivation by HHP **(Guimarães *et al*., 2002; Durães-Carvalho *et al*., 2012)**.

A relevant concern related to the use of HHP for bacterial inactivation is the resistance of some microorganisms to this treatment. Indeed, the development of

resistance to high pressure has important implications for the use of pressure technology in the inactivation of nonpathogenic and pathogenic *E. coli* strains in food preservation methods **(Hauben *et al*., 1997)**. **Vanlint *et al*. (2012)** described the isolation of extremely HHP-resistant mutants of *E. coli* MG1655 strain (resistant up to 2 GPa) by applying a selective enrichment approach based on consecutive cycles of increasingly severe HHP shocks. These authors subsequently showed that the capacity to develop extreme resistance to HHP was mainly related to species of *E. coli*, although not all isolates shared this characteristic **(Vanlint *et al*., 2012)**. According to these authors, once acquired, HHP resistance was apparently a stable trait and showed that *E. coli* strains readily survive to 800 MPa.

According to **Hauben *et al.* (1997)**, if barotolerant strains can grow under commercial pressurization processes then they represent a serious threat to the safety and stability of pressure-processed foods. For these reasons, we believe that the combination of HHP at relatively moderate hydrostatic pressure (250 MPa) and aqueous pomegranate extract could be a promising alternative for avoiding the selection of pressure-tolerant pathogenic strains and for inactivating important foodborne pathogens such as STEC O157:H7 strain.

CONCLUSIONS

Our results showed that bacterial inactivation was drastically increased when HHP treatment (250 MPa at 25°C during 60 minutes) was applied together with pomegranate peels extract, leading to total inactivation of STEC O157:H7 in contaminated milk. Considering the promising future of HHP technique in food industry and that no viable bacteria were detected after the application of combined treatment, we consider that this process has potential for reduction of bacterial load in food contaminated by STEC O157:H7. The synergistic effect of moderate pressure and natural extract of pomegranate on bacterial inactivation, make it economically attractive for industry and may prevent the selection of barotolerant pathogenic strains. Additional studies will be needed to improve the efficiency of this treatment and to achieve the neutralization of biological activity of the toxins produced by this important pathogen.

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MICROBIAL BIOSURFACTANTS: METHODS FOR THEIR ISOLATION AND CHARACTERIZATION

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ABSTRACT

Biosurfactants are amphiphilic molecules produced by microorganisms. They are exopolymers of low and high molecular weight, capable to emulsify and decrease the surface tension water. Recently, the interest for its production has increased, because of their environmental, industrial, and pharmaceutical applications, and others. However, in order to obtain a biosurfactant, it is necessary to research the producer strain of biosurfactant, its optimal conditions of incubation and carbon sources. Once recovered the biosurfactants, it is required to know their biochemical composition, for this purpose we have developed sensitive and analytical techniques to discover the wide diversity of biosurfactants The aim of this review is to report fundamentals for methods and techniques for qualitative and cuantitative analysis of microbial biosurfactants. It is aimed at students, biotechnologists and other experts interested in exploring tensoactive molecules of glycolipidic nature. It also contains detailed information about the solids culture media used (specific and selective), different methods to recover these expolymers: acid precipitation and organic solvent extraction. Finally in this review it includes the most innovative and comprehensive methods used for chemical characterization of biosurfactants: spectroscopy UV/Vis, infrared spectroscopy, nuclear magnetic resonance, gaseous and liquid chromatography and mass spectrometry.

Keywords: Rhamnolipids, surface tension, emulsifiers, biotechnological applications

INTRODUCTION

The biosurfactants (BS) are extracellular compounds produced mainly by fungi and bacteria **(Banat *et al*., 2000)**. These molecules have emulsificant and dispersant properties, they achieve reduce water surface tension from 72 to 25 mN/m approximately **(Supaphol *et al*., 2011)**. The microbial biosurfactants can enhance bioavailability and biodegradability of low solubility compounds **(Chrzanowski *et al*., 2012)**, for this reason they have been applied in bioremediation processes of polluted environmental sites with heavy metals, organophosphate pesticides and hydrocarbon total petroleum **(Banat *et al*., 2000; Ortíz-Hernández *et al*., 2001; Yañez-Ocampo *et al*., 2009; Yañez Ocampo *et al*., 2011)**. In addition, BS have applications in the alimentary, pharmaceutical and cosmetical industry **(Singh *et al*., 2007; Sajna *et al*., 2013)**.

For the biotechnological production of BS there are two stages to consider, first microbial strains potentially producers of BS must be explored, isolated and adapted by selective and differential media **(Konishi *et al*., 2015)**, in this stage it is important to carry out several assays in order to know the emulsion index as well as tensoactive, hemolytic and dispersant activity **(Cassidy and Hudak, 2001; Kitamoto *et al*., 2002)**. In addition, it is required the research of the optimal culture conditions in liquid medium such as pH, agitation speed, temperature and selection of the carbon source for the culture medium. In this sense nowadays there is an interest for using low cost carbon sources such as soja oil, corn oil used, glycerol, lacto serum, coffee waste, and others hydrophobic compounds by example n-decane, n-tetradecane, paraffin **(Abbasi *et al*., 2012; Abbasi *et al*., 2013)**. The second stage refers for their production and industrialization; the costs can be for approximately 60% of the total production cost, because of the unitary operations to obtain BS **(Makkar *et al*., 2011; Reis *et al*., 2013)**. The BS are extracellular products, therefore techniques used to separate and recover, depend of its biochemical nature and the selection of the kind method to isolated them **(Sen and Swaminathan, 2005)**. The most common techniques are acid precipitation and extraction with organic solvents.

After extraction it is necessary to purify and characterize the tensoactive, this is achieved by applying different methods; chromatographic and spectroscopic **(Smyth *et al*., 2010; Makkar *et al*., 2011; Thavasi *et al*., 2011; Kuyukina *et al*., 2013)**. The optimal use of new analytic instruments, requires basic comprehension and knowledge of principles for these specific measurement systems, in order to obtain a highly tensoactive molecule.

About this last topic the molecular characterization of BS, it is known that they are amphiphilic molecules of low molecular weight, mainly glycolipids and lipopeptids, the polar region or hydrophilic is a carbohydrate (rhamnose, trehalose, mannose) and the non polar region or hydrophobic is a hydrocarbonated chain of long variable (fat acids saturated and unsaturated). Figure 1 show the principal chemical structures of BS best studied **(Banat *et al*., 2000; Banat *et al*., 2010; Abdel-Mawgoud *et al*., 2010; Müller *et al*., 2012)**.

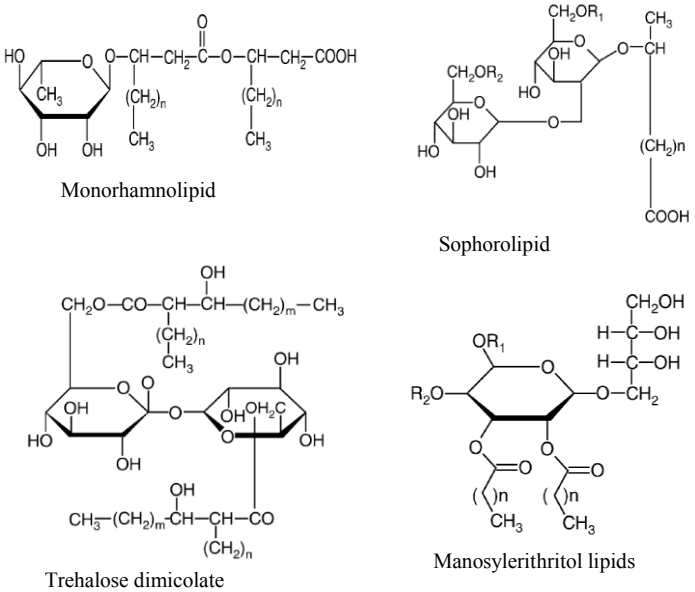

Figure 1 Chemical structures of the best studied microbial biosurfactants **(Banat *et al*., 2010)**

Several species of *Rhodococcus* sp. can produce trehalolipids, also some yeast as *Candida* sp. can synthesize manosylerithritol lipids, *Bacillus subtilis* produce a BS known as surfactin that currently is used for industrial purposes with pharmaceutical and alimentary applications **(Al-Bahry *et al*., 2013)**. The aim of this paper is to contribute with a review about the recent methods to select and adapt strains producers of biosurfactants, as well as its analytical spectroscopic methods and chromatographic to elucidate quantitative and qualitatively its structure.

FUNDAMENTALS FOR ISOLATION OF MICROORGANISMS PRODUCERS OF BIOSURFACTANTS

In several environments like sea, mangrove sediments or soil polluted with oil hydrocarbon or pesticides, the microorganisms produce biosurfactants, mainly when they are exposed to low solubility carbon sources **(Souza *et al*., 2014)**. In vitro conditions, is known that an excess in carbon source (by example glucose, glicerol or frying oils used) and stress with low levels of nitrogen, promotes rhamnolipids production **(Lee *et al*., 2008; Arutchelvi *et al*., 2011; Kryachko *et al*., 2013)**. The group of *Pseudomonas* sp., are producers of mono and dirhamnolipids, their biosynthesis pathway involves rhamnosyltransferases RhlB y RhlC mainly. Several reports indicate that rhamnolipids production in *P. aeruginosa* is strongly controlled by genetic regulation (transcriptional and postranscriptional) of the rmlBDAC and rhlAB operons called quorum sensing, it is a bacterial communication system characterized by the secretion and detection of signal molecules – autoinducers – within a bacterial population **(Soberón-Chávez *et al*., 2005; Müller *et al*., 2012; Reis *et al*., 2013; Cortés-Sánchez *et al*., 2013)**.

QUALITATIVE METHODS FOR BACTERIAL BIOSURFACTANTS DETECTION

The most reported assays for detection of bacteria producers of biosurfactants, mainly of glycolipidic nature are described briefly in this section.

Agar hexadecyltrimethylammonium bromide (CTAB) assay

This method can detect the production of anionic biosurfactant, rhamnolipids specifically. The medium used for this purpose is an agar based on mineral salts, a low solubility carbon source (v. gr. glycerol, vegetable oil, hydrocarbon), blue methylene and CTAB. The CTAB is a cationic salt that reacts with the rhamnolipid, this complex (CTAB-rhamnolipid-blue methylene) can be visualized by presence of traslucid halo around the colony growth on the agar plates (Figure 2) **(Chandankere *et al*., 2013)**. CTAB can be replaced with N-cetylpyridinium chloride, benzethonium chloride or alkylbenzyldimethylammonium chloride, $CaCl_2$ or $AI(OH)_3$ also can be used instead of CTAB. The blue methylene can be substituted for several dyes like fuchsin, safranin or dichlorphenolindophenol **(Youssef *et al.*, 2004)**.

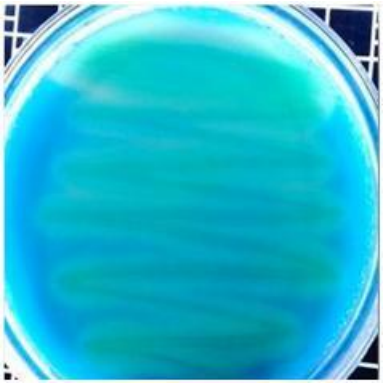

Figure 2 Halo around the bacterial growth agar with methylene blue

Hemolytic activity assay

This method can visualize indirectly the biosurfactants production by inoculating of bacterial strain on a blood agar plate (5 % v/v), incubate to 30 ºC for 24-48 h. The presence of traslucent halos around to growth on the agar plates indicates BS presence with hemolytic activity **(Hassanshahian, 2014)**. However, some bacteria release proteases, or beta-hemolysins, so this test often leads to a positive false result. Besides, not all biosurfactants have hemolytic activity, for this reason it is necessary to perform more tests to confirm BS production **(Youssef *et al*., 2004; Zhang *et al*., 2012)**.

Drop collapse test

This method is quick and easy to detect of BS presence in a sample of bacterial liquid medium culture. By applying a drop of cell free supernatant (BS crude), on a set of hydrophobic phases (v. gr. mineral oil, soja oil, motor oil) it is possible to see the collapse of the oil drop (Figure 3). Additionally it is necessary to run several positive controls, using synthetic surfactants as SDS or tween 80 and try different concentrations. For more details about this assay it is recommended to consult to **Tugrul and Cansuna, (2005); Abdel-Mawgoud *et al*., (2011)**.

Figure 3 *Pseudomonas aeruginosa* supernatant on the oily surface it is collapsed **(Tugrul and Cansunar, 2005)**

Oil displacement test

This technique consist in adding 50 mL of destilled water in a Petri dish, 100 µL of oil and from 5 to 30 µL of cell free supernatant **(Affandi *et al*., 2014; Hassanshahian, 2014)**. The tensoactive activity can be measured with the diameter of the oil displaced after addition of BS. The increased of the diameter is directly related to a high tensoactive activity. This test provides indirect information about biological compounds with tensoactive activity. Figure 4 shows a representative study.

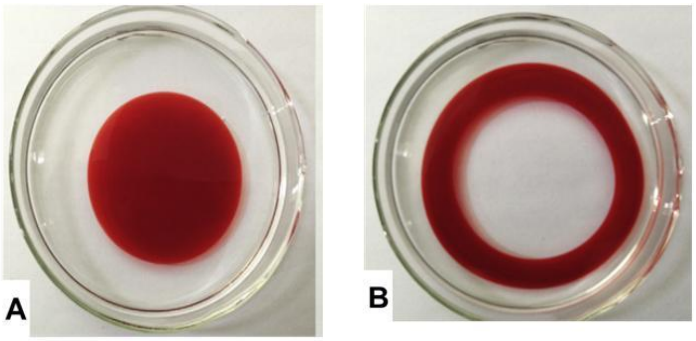

Figure 4 Oil drop displacement A) red oil drop before the experiment B) after applying cell free supernatant (5µL) **(Liu *et al*.,2015)**

Emulsion index (IE_{24})

The emulsification index consist in adding in an screw-cap test tube a mixture (1:1 v/v) of a hydrocarbon (motor oil, corn oil, cotton oil, n-hexadecane, diesel or kerosene) **(Dubey *et al*., 2012)** and cell-free culture broth, after a vigorous homogenizing for two minutes in a vortex, the mixture is left to repose for 24 h to see a stable emulsion layer (Figure 5) **(Ayed *et al*., 2015)**. The emulsification index is calculated by dividing the height of the emulsion layer by the total height of the mixture and multiplying by 100 **(Hassanshahian, 2014; Luna *et al*., 2015)**. In this test, it is also necessary to perform the same positive controls cited above.

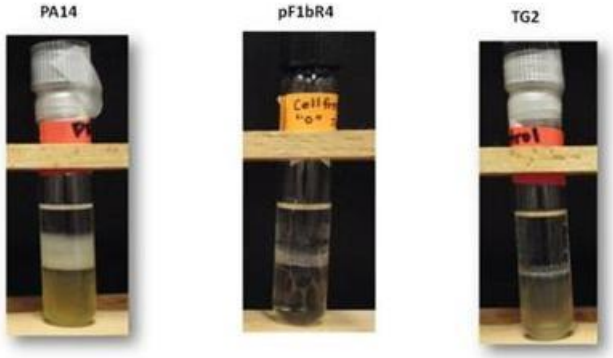

Figure 5 Emulsification of hexadecane by *P. aeruginosa* PA14, *E. coli* pF1bR4 and *E. coli* TG2 **(Arutchelvi *et al*., 2011)**

Determination of Surface tension

This parameter is measured using a digital tensiometer with a Du Noüy ring or Wilhelmy plate. Both methods quantify the strenght required to separate the ring or platinum plate from the liquid surface. The strength is reported in mN/m, this indicates the surface tension the liquid containing BS. Information detailed about specific procedures are available in **Abdel-Mawgoud *et al*. (2011); Burgos-Díaz *et al*. (2013); Xiao *et al*. (2013); Ayed *et al*. (2015)**. A typical parameter for studying biosurfactants is the Critical Micellar Concentration or CMC, who means the concentration of biosurfactant required for micella formation and also reach the lower value of surface tension. Table 1 shows CMC values for various biosurfactants, organized by ascending order, from lowest to highest value. The ideal biosurfactant must have a low value of CMC and also achieve a minimum surface tension **(Mao *et al*., 2015)**.

Table 1 Values of CMC and surface tension published for biosurfactants produced by microorganisms

Microorganism	Biosurfactant	CMC (g/L)	Surface tension (mN/m)	Reference
Bacillus subtilis	Surfactin	0.017	27.2	**Sen and Swaminathan, 2005**
Bacillus subtilis PT2	Surfactin	0.025	26.4	**Pornsunthorntawee *et al*., 2008**
Pseudomonas fluorescens BD5	Lipopeptid	0.072	31.5	**Janek *et al*., 2010**
Pseudomonas aeruginosa SP4	Monorhamnolipid	0.120	28.3	**Pornsunthorntawee *et al*., 2008**
Rhodococcus spp. MTCC 2574	N/I	0.120	30.8	**Mutalik *et al*., 2008**
Sphingobacterium sp.	Phospholipid/ Lipopeptide	6.3	22	**Burgos-Díaz *et al*., 2011**
Streptococcus thermophilus	Glycolipid	20	36	**Rodrigues *et al*., 2006**

N/I= not identified, CMC= Critical Micellar Concentration

EXTRACTION METHODS OF BIOTENSOACTIVE COMPOUNDS

After incubation time in a medium of biosurfactants production, it is necessary to select a method for their extraction. In this section, the most common methods used for this purpose are reported.

Extraction by centrifugation

Through centrifuge force, is possible to separate biomass from the culture medium to obtain a cell-free supernatant, which contains total biosurfactants. Table 2, shows different speeds centrifugation to separate the biomass from the culture broth and obtain the supernatant with total biosurfactantes.

Table 2 Speed centrifugation to extract total biosurfactants from biomass in the culture medium

Speed *g*	Centrifugation time (min)	Temperature (ºC)	Type of biosurfactant	Reference
5000	20	4	Lipopeptide	**Rufino *et al*., 2014**
7,656	10	N/A	Trehalolipid	**Bajaj *et al*., 2014**
8,000	15	4	Lipopeptide and Glycolipids	**Burgos-Díaz *et al*., 2011**
8,643	20	N/A	Lipopeptide	**Sarafin *et al*., 2014**
9,690	30	4	Rhamnolipids	**Aparna *et al*., 2012**
10,000	15	4	Rhamnolipids	**Abbasi *et al*., 2012**
10,800	20	4	Ochrosin	**Kumar *et al*., 2014**
10,956	10	N/A	Surfactin	**Liu *et al*., 2015**
11,952	20	4	Lipopeptide	**Xia et al., 2014**
11,952	10	4	Rhamnolipids	**Bharali *et al*., 2013**
11,952	15	4	Rhamnolipids	**Zou *et al*., 2014**
14,000	5	N/A	Rhamnolipids	**Costa *et al*., 2011**
20,217	20	4	Glycolipopeptid	**Jain *et al*., 2013**

N/A= data not available

Extraction by acid precipitation

Once obtained the cell-free supernatant, it is necessary to recover the mixture of biosurfactants, for this purpose a method commonly used is the acid precipitation. The supernatant must be acidified with 2 N HCl until pH 2.0 and is kept overnight at 4 ºC. The precipitate obtained is recovered by centrifugation and the pellet is washed with acidic water (pH 2.0 with HCl) and then washed with alkaline water (pH 11.0 with NaOH) to achieve a final pH 7.0. The precipitate is dried with heat **(Salleh *et al.*, 2011)**. Table 3 shows several reports about the extraction of biosurfactants with a mixture of both methods: acid precipitation and extraction with solvent.

Extraction by organic solvent

This technique is used in combination with acid precipitation to obtain higher yields of extraction. The supernatant is acidified in separatory funnel with HCl to pH = 2.0, after equal volume of solvent is added, both liquids are agitated, allowing to stand until observe two separate phases, the organic phase is recovered and placed it in a rotary evaporator until observe an extract of brown colour. The organic solvent extraction after acid precipitation is the most applied method to recover approximately 90 % of BS **(Gusmão *et al*., 2010; Salleh *et al*., 2011)**. The solvents used in the extraction can be recovered and reused, this represents good laboratory practice.

Rufino et al. **(Rufino *et al*., 2014)** reported BS recovering from the culture of *Candida lipolytica* UCP 0988, using the mixture chloroform/culture broth (1:1 v/v), with a yield of 8.0 g/L of tensoactive. It has also reported BS extraction with a mixture ethyl acetate/ methanol (8:1 v/v) and chloroform/methanol (2:1) v/v recovering 10 g/L BS crude **(Burgos-Díaz *et al*., 2011; Affandi *et al*., 2014)**.

Extraction by Lyophilization

Lyophilization is also a methodology used to recover BS. In this technique, the first step is to obtain a cell-free supernatant by centrifugation, after the exopolymer is precipitated by adjusting pH = 2.0 with chlorhydric acid overnight at 4 ºC. The pellet precipitated is recovered by centrifugation and it is resuspended in distilled alkaline water (pH = 8.0) and then is lyophilized to obtain a brown powder. Through this method, **Al-Bahry *et al*. (2013)** recovered 2.29 g/L of BS from a culture of *Bacillus subtilis* B20, also **Xiao *et al*. (2013)** produced BS with *Klebsiella* sp., in their research, were recovered 10.1, 5.1, 3.25, 3.1, 2.75 and 2.62 g/L of BS produced with starch, sucrose, xylose, galactose, glucose and fructose respectively.

SPECTROPHOTOMETRIC METHODS TO QUANTIFY BIOSURFACTANTS

Spectrophotometric methods are used to determine the presence of biosurfactants in either the culture medium or with the compounds extracted. The presence of sugars in the BS can be identified by different methods spectrophotometrics such as anthrone, phenol-sulfuric, orcinol and DNS **(Smyth *et al*., 2010; Abdel-Mawgoud *et al*., 2011)**. However, interferences from chemicals and carbon sources can result in inaccurate results and therefore should only be used as a rough indicator of biosurfactant production.

Anthrone Assay

This assay can be used for all types of glycolipid biosurfactants, it detects the amount of carbohydrate present. With the anthrone reagent previously prepared, add 200 µL of cell-free supernatant and 1000 µL of anthrone, heat the sample for 9 min, then measure by absorbance at 625 nm **(Smyth *et al*., 2010)**. The anthrone assay can be used to detect of glycolipid in the culture broth.

Phenol-sulphuric acid assay

The phenol-sulphuric acid reagent is widely used as a chemical method for the measurement of the sugars of polysaccharides, glycoproteins and glycolipids. The method is sensitive for determining small quantity of sugars in biological samples. Add 2 mL of supernatant in the test tube, add 1 mL of phenol (5%) and 5 mL of sulfuric acid concentrated, measure after 30 min at 490 nm **(Rufino *et al*., 2014)**.

Orcinol assay

This colorimetric assay is based on the reaction of orcinol (1,3-dihydroxy-5-methylbenzene) and the sugar moiety under acidic conditions and high temperature to produce a blue-green colored dye whose absorbance can be measured at 665 nm and used to roughly quantify the glycolipid concentration in the sample **(Smyth *et al*., 2010; Abdel-Mawgoud *et al*., 2011)**.

DNS assay

Is based on a redox reaction between 3,5 dinitrosalicilic acid and reducers sugars in supernatant free cell culture. In order to determine sugar concentration, 3 mL sample with 5 mL of chlorhydric acid (HCl 6 N) are mixed in glass tubes and heated to 65 ºC by 10 min after it is cooled and neutralized with sodium hidroxide (NaOH), subsequently reactant DNS is added. Absorbance is measured in a spectrophotometer at 540 nm; is necessary to build a standard curve, the result is expressed in g/L **(Smyth *et al*., 2010; Abdel-Mawgoud *et al*., 2011)**.

Table 3 Extraction methods for recover biosurfactants by using acid precipitation and organic solvents

Acid precipitation conditions	Solvent used	Microorganism producer of biosurfactant	Yield (g/L)	Reference
H_2SO_4 1 M pH 2.0	Chloroform/ Methanol (2:1v/v)	*Pseudomonas* sp. 2B	4.97	**Aparna *et al*., 2012**
HCl 6 M pH 2.0 Overnight at 4 ºC	Methanol	*Bacillus subtilis* BS-37	0.585	**Liu *et al*., 2015**
HCl 2 N pH 2.0	Ethyl acetate	*Ochrobactrum* sp. BS-206 (MTCC 5720)	0.28	**Kumar *et al*., 2014**
HCl 2 N pH 2.0 Overnight at 4 ºC	Ethyl acetate	*Rhodococcus* sp. IITR03	N/A	**Bajaj *et al*. 2014**
HCl 6 M pH 2.0 Overnight at 4 ºC	Methanol	*Candida sphaerica* UCP0995	9	**Luna *et al*., 2015**
HCl 6 N pH 2.0	Chloroform/ Methanol (65:15 v/v)	*Kocuria marina* BS-15	0.00197	**Sarafin *et al*., 2014**
HCl 6 N pH 2.0 Overnight at 4 ºC	Ethyl acetate	*Pseudomonas aeruginosa* MA01	12	**Abbasi *et al*., 2012**
HCl 6 N pH 2.0 Overnight at 4 ºC	Ethyl acetate	*Pseudomonas aeruginosa* OBP1	N/A	**Bharali *et al*., 2013**
HCl 6 N pH 2.0 Overnight at 4 ºC	Chloroform/ methanol (2:1 v/v)	*Pseudomonas* sp.	5.2	**Silva *et al*., 2014**
N/A	Chloroform	*Candida lipolytica* UCP 0988	8	**Rufino *et al*., 2014**
N/A	Ethyl acetate/ methanol (8:1 v/v)	*Sphingobacterium detergens*	0.466	**Burgos-Díaz *et al*., 2013**

N/A= data not available

METHODS AND TECHNIQUES FOR BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF BIOSURFACTANTS

The analytical chemistry employs several quantitative and qualitative methods to elucidate the chemical structure of organic compounds. The qualitative method reports the identity of the atomic species and functional groups of the sample. The quantitative method gives the numeric information about the relative quantity in one or several components.

The main methods to study the biochemical composition and molecular characterization of BS are described next, such as Fourier transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR), also chromatographic methods like thin layer chromatography (TLC), high performance liquid chromatography (HPLC) **(Swaathy *et al*., 2014)** coupled with a light scattering detector (ELSD), mass spectrometry coupled with high performance liquid chromatography (HPLC–MS) or gas chromatography (GC–MS) **(Ribeiro *et al*., 2012)**. Table 4 reports the methods used to characterize BS, mainly rhamnolipids.

Table 4 Methods for the qualitative and quantitative study of biosurfactants

Instrumental method	Type of biosurfactant	Reference
Infrared Spectroscopy (IR)	Glycolipids, rhamnolipids, xylolipid, lipopeptids	**Jain *et al*., 2013; Vecino *et al*., 2013; Chandankere *et al*., 2013; Jain *et al*., 2012; Sharma *et al*., 2015; Nalini and Parthasarathi, 2014; Jara *et al*., 2013; Aparna *et al*., 2012; Pantazaki *et al*., 2011; Ibrahim *et al*., 2013; Ismail *et al*., 2013**
Nuclear Magnetic Resonance (NMR)	Sophorolipid, xylolipid	**Konishi *et al*., 2015; Sharma *et al*., 2015**
Thin layer chromatography (TLC)	Lipopeptids, glycolipids	**Sharma *et al*., 2015; Burgos-Díaz *et al*., 2011; Xia *et al*., 2014; Pedetta *et al*., 2013**
High Performance Liquid Chromatography (HPLC)	Rhamnolipids, xylolipid	**Sharma *et al*., 2015; Pantazaki *et al*., 2011; Compaoré *et al*., 2013**
Column Chromatography (CC)	Lipopeptids ,glycolipids	**Burgos-Díaz *et al*., 2011**
Gas chromatography (GC)	Xylolipid	**Sharma *et al*., 2015**
Mass spectrometry (MS)	Rhamnolipids, xylolipid	**Gudiña *et al*., 2015; Sharma *et al*., 2015; Pantazaki *et al*., 2011**

Infrared spectroscopy

Nowadays the infrared spectroscopy is used as a technique to identify organic molecules like biosurfactants, through the vibrations and frequences characteristics of their chemical bonds, in the interval of infrared spectrum. The infrared spectroscopy is based on the infrared radiation absorption by molecules in vibration. Every type of chemical bond absorbs the infrared light at a specific frequence, so it is possible to know what kind of functional groups have the molecules in study. For example, the C-H bonds are localized in the spectrum of 4000-2500 cm^{-1} of wavelength **(Chadwick *et al*., 2014)**.
Each molecule has a typical infrared spectrum like a finger print, so analyzing several wavelengths that a substance absorbs in infrared zone, it is possible to know the functional groups that the glycolipid has **(Madsen *et al.*, 2011; Li *et al*., 2014)**. It is based on the relatively broad IR absorption bands corresponding to various hydroxyl, ester, and carboxylic groups present in glycolipids. This method has been used for the quantification of complex RL mixtures, but it suffers from interferences by other constituents in the medium and of changes in pH **(Rikalović *et al*., 2012)**.

Nuclear Magnetic Resonance

The previously reported methodologies allow the identification of molecular structure to quite a high extent though not completely. To achieve a full structural determination, NMR needs to be utilized and it is the most powerful method, this is able to identify functional groups as well as the position of linkages within the carbohydrate and lipid molecules. Using a series of NMR experiments the exact location of each functional group can be obtained and information about the structural isomers is also possible **(Smyth *et al*., 2010)**.
Nuclear magnetic resonance (NMR) spectroscopy is a powerful tool for structural elucidation, which is obtained in 1D and 2D experiments. In organic chemistry, the main tools for structural elucidation are 1H and 13C NMR spectra associated with 2D experiments, such as 1H–1H COSY, 1H–1H TOCSY, 1H–13C HMQC, 1H–13C HMBC and NOESY. The glycolipids should be dissolved in dueterated chloroform and a series of 1D (1H and 13C) and 2D (such as COSY, ROSY, HMQC and HMBC) experiments should be carried out by NMR. Specific details with regard to the results for rhamnolipids, sophorolipids, trehalose lipids and mannosylerythritol lipids can be obtained from Smyth et al. **(Smyth *et al*., 2010)**.

Chromatography

This method is used to separate organic compounds from complex mixtures. Chromatography is based in a mobile phase which transport the sample, the mobile phase can be gas or liquid, then sample is transported into a stationary phase is a column, every compound of the mixture in the sample is distributed. The compounds of the sample are separated, by this process the biosurfactants can be analized qualitatively or quantitatively **(Sen and Swaminathan, 2005; Abbasi *et al*., 2013)**.

Thin layer chromatography

Thin layer chromatography (TLC) is a simple method allowing detection of glycolipids and can also provide information on possible structural types of glycolipids present. TLC detection should be carried out before purification procedures to determine the presence of glycolipids and can also be used to determine purity after purification steps **(Smyth *et al*., 2010; Abdel-Mawgoud *et al*., 2011)**.
TLC can be used as a preliminary method for evaluation and/or screening of SLs production. A stationary phase of silica GEL 60 or silica GEL F254 is frequently used in association with chloroform/ methanol/water mobile phase. For detection of glycolipids bands by TLC, several staining solutions in association with heat have been used and examples are anthrone-naphtol and p-anisaldehyde **(Ribeiro *et al*., 2012)**.

Gas chromatography

Specifically rhamnolipids cannot be directly analyzed by gas chromatography (GC) because of their relatively high molecular weight. Prior to analysis, rhamnolipids are hydrolyzed with acid, their acid groups are modified into methyl esters, then rhamnose and various 3-hydroxyfatty acids can be analyzed, identified and quantified using flame ionization detection by GC **(Abdel-Mawgoud *et al*., 2011)**.

High Performance Liquid Chromatography (HPLC)

HPLC is a method that allows the separation of glycolipids **(Compaoré *et al*., 2013)**. This method is especially well-suited for analysis of rhamnolipids, it is generally performed using C8 or C18 reverse-phase columns with a water/acetonitrile gradient **(Hassanshahian, 2014)**. HPLC-UV can also be used for analysis when the test compounds have been derivatised to p-bromophenacyl esters **(Smyth *et al*., 2010)**. HPLC-UV and HPLC-ELSD both require comparison with retention times of standards to allow identification of the structure, however, the presence of isomers cannot be detected by Gas chromatography.

Mass spectrometry

The mass spectrometry analysis can be used to identify the chemical structures of biosurfactant mixtures **(Rikalović *et al*., 2012)**. Electrospray Ionization (ESI) is used to ionize BS prior to mass analysis. The structures of the mono- and di-rhamnolipids were confirmed by electrospray tandem mass spectrometry (ESI–MS/MS). The molecular weight of the pseudomolecular ion [M-H] can be directly obtained. This provides some information on the nature of the BS congener eluting from the column at that retention time **(Abdel-Mawgoud *et al*., 2011)**. Several strains of *P. aeruginosa*, produce mixtures of rhamnolipids of different congeners, being the most common L-rhamnosyl-bhydroxydecanoyl- b-hydroxydecanoate (Rha-C10-C10) and Lrhamnosyl- L-rhamnosyl-b-hydroxydecanoyl-b-hydroxydecanoate (Rha-Rha-C10-C10) **(Aparna *et al*., 2012)**. Other congeners frequently found include mono- and di-rhamnolipids with acyl chains containing 8, 10, 12 or 14 carbons, mostly saturated, and, less often, containing one or two double bonds, as well as with only one b-hydroxy fatty acid [42, **(Gusmão *et al*., 2010; Ibrahim *et al*., 2013; Janek *et al*., 2013; Hoškova *et al*., 2013)**.

Table 5 Chemical composition of rhamnolipid mixture produced by *Pseudomonas* sp. 2B culture determined by MS analysis **(Aparna *et al*., 2012)**

Rhamnolipid congeners	Pseudomolecular ion (*m/z*)
Rha-Rha- C_8-C_{10}	621.0
Rha-Rha- C_{10}-C_{10}	650.0
Rha-Rha- C_{10}-C_{12}	678.0
Rha-C_{10}-C_{10}	505
Rha- C_{10}-C_{12}	532
Rha-C_{10}	333
Rha- C_{10}-C_{10}	504
Rha-Rha- C_{12}-C_{10}	678
Rha-Rha- C_{10}	479

CONCLUSION

The biotechnological production of biosurfactants can be feasible with use of carbon sources low cost like organic wastes from agriculture or vegetable used oils. The biosurfactants production to an industrial scale remains challenge, to substitute the synthetic surfactants. Despite their environmental advantages and equal performance, commercialization of these molecules remains a challenge. The latter issue can partially be tackled by screening for the research of better producers and optimizing the fermentation process. In order to reduce cost of production of biosurfactants, there are several methods to extract them from culture medium, like centrifugation or acid precipitation. For environmental applications of biosurfactants, it requires only biosurfactant crude from a free cell culture. For scientific and patent creation purposes it is necessary to have a complete characterization, therefore, spectroscopic, chromatographic and mass spectrometry methods are accurate powerful tools to elucidate novels biosurfactants.

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SPECIFICITY OF ADHESION OF *BACILLUS SUBTILIS* IMV B-7023 TO ABIOTIC AND BIOTIC SURFACES

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ABSTRACT

The adhesion of *Bacillus subtilis* strain IMV B-7023 cells to surfaces of different nature was studied. It was demonstrated that the sorption of cells to abiotic solid materials was considerably governed by the bacterial motility which depended on the growth phase. An important role in the interaction process involving cells and biotic surfaces was attributed to bacterial lectins. It was determined that surface lectins of *B. subtilis* specific for D-glucosamine chloride and, in a lesser degree, to D-glucuronic and D-galacturonic acids were also active participants of specific adhesion of bacteria to plant roots. The blockage of these lectins with D-glucosamine chloride caused bacterial adhesion to the plant roots by 26%. Thus different mechanisms may be involved into the adhesion process of bacterial cells to abiotic and biotic surfaces.

Keywords: *Bacillus subtilis*, adhesion, surface lectins, solid materials, biotic surface

INTRODUCTION

The main part of microbiota in soil is known to function due to its contact with soil particles, vegetable debris and their roots (**Costerton et al. 1985**). The process of bacteria adhesion is one of the primary stages of their contact interaction with solid surfaces which then results in the multiplication of cells on surfaces and their colonization (**Zviagintsev et al. 1977**). According to the literature data, the main factors, determining the adhesion of microorganisms to abiotic surfaces, are physical and chemical properties of contact surfaces, namely, their charge and hydrophobicity (**Kurdish 2010**).

During the adhesion of bacteria to biotic surfaces, plant roots in particular, an important role is played by specific interaction between certain sites of plant root cells and microorganisms. This process is grounded on the principle of complementarity of interacting structures where the leading role is attributed to the lectin-carbohydrate interaction (**Nikitina 2001; Karpunina 2005**). The literature data testify to the presence of lectins on the surface of cells of spore-forming microorganisms (**Karpunina 2005; Podgorsky et al. 1992; Sharon and Lis 2007**). The specificity of the lectins of *Bacillus subtilis* IMV B-7023 which is a component of a highly efficient complex bacterial preparation used in agriculture and the lectins impact on the adhesion of cells to plant roots has not been studied yet. Our work was aimed at the comparative study of the specificities of *B. subtilis* IMV B-7023 adhesion to abiotic and biotic surfaces, the determination of the presence and specificity of lectins of these bacteria and their impact on the adhesion process to the surface of plant roots. The determination of the regularities of this process will help to understand the specificities of bacteria-plants interactions when the microorganisms are introduced into agroecosystems.

MATERIALS AND METHODS

Microorganisms, nutrient media and culture conditions

The object of the research was a highly efficient strain of phosphate-mobilizing bacteria *Bacillus subtilis* IMV B-7023 selected at the Department of Microbiological Processes on Solid Surfaces, Zabolotny Institute of Microbiology and Virology, National Academy of Science of Ukraine (Patent No. 54923A).

The hydrophilic particles of glass, representing the wide-spread in different types of soils silicates, were used as the abiotic surfaces. The roots of cucumber plants of Konkurent cultivar were used as a model of biotic surface, as the seeds of this cultivar are easy to sterilize and germinate.

The cultivation of bacteria was performed for 18–72 h at 28°C under periodic conditions in the shaker (240 rpm) using 750 mL Erlenmeyer flasks with 100 ml of the medium as follows (g/L): $(NH_4)_2SO_4$ – 0.5; $MgSO_4 \cdot 7H_2O$ – 0.3; NaCl – 0.3; KCl – 0.3; $MnSO_4 \cdot 5H_2O$ – 0.001; $FeSO_4$ – 0.001; $CaCO_3$ – 0.5; calcium glycerophosphate – 2.0; glucose – 10.0; pH of the medium 6.8–7.0. The medium was inoculated with 1 mL of bacterial suspension containing 10^9 cells/mL.

Determination of adhesion of bacteria

The study of bacterial adhesion to the abiotic surface involved the use of chemically pure glass flasks with the inside diameter of 16 mm and the height of 180 mm, which were introduced with 15 g of chemically pure 1–2 mm glass particles each. Each tube was added with 6 ml bacterial suspension to ensure complete covering of the carrier layer. After the contact with the adsorbent for 2 h the suspension was poured off with subsequent determination of the difference in its optic density prior to the interaction with the carrier and after it. After making obtained suspension up to the initial volume itse optic density was determined using the photocolorimeter KFK-2 MP (Zagorsk,Russia), (wavelength 540 nm, cuvette 5 mm). The number of bacteria in suspensions prior and after their interaction with the carrier was defined by the curve of optical density versus the number of cells (**Gordienko et al. 2009**) and the number of cells, sorbed per 1 g of glass granules, was estimated.

The bacteria adhesion to cucumber roots was studied as follows. Cucumber seeds (*Cucumis sativus* L.) of Konkurent cultivar were treated with the mixture of ethyl alcohol and 50 % hydrogen peroxide in 1:1 ratio for 30 min, washed thrice with the sterile physiological solution and cultivated in potato agar in Petri dishes for 3–5 days (depending on the batch). For preparation of the given medium 200 g of potato have been boiled in 1L water, pH 7.0 and 20 g agar was added. The roots of sterile sprouts obtained were cut into segments of 1–1.5 cm and introduced in portions of 100 mg into the 10 cm long tubes with the diameter of 20 mm. Washed with Na–K phosphate buffer (pH 7.0) of the bacterial suspension (3mL) containing at least 10^8 cells/mL was added to the tubes containing the cucumber roots. The tubes were placed in the shaker (type W-4, Premed, Poland, 130 rpm) and incubated at room temperature (19–21°C) for 1 h. The roots were washed thrice with the sterile physiological solution at the microshaker type ML-1 (Premed, Poland), homogenized in the mortar and made up to the volume of 10 ml using the physiological solution. The number of adhered living cells was determined by the number of colonies (colony-forming units, CFU) growing on the agarised potato medium in Petri dishes after the homogenate from ten-fold dilutions was plated and expressed as CFU per 1 g of wet weight of the roots. All the experiments were conducted at least thrice in three replicas.

The motility of bacteria was estimated in a “hanging drop” using microscopy (**Yegorov 1959**), the hydrophobicity of their surface was determined by the

distribution of cells in the "suspension of bacteria-n-hexadecane" system (**Rosenberg and Kjelleberg 1986**).

Determination of lectins in *Bacillus*

The presence of lectins in *Bacillus* was determined by the results of hemagglutination reaction (HAR) in two-fold dilutions in series in sterile U-like 96-well polystyrene microplates at room temperature using trypsinized rabbit erythrocytes, fixed with glutaraldehyde (**Lutsyk et al. 1980)**. The control was selected as 2 % suspension of erythrocytes in the physiological solution.
The carbohydrate specificity of surface lectins of bacilli was studied after 50 h of their cultivation in the abovementioned medium according to the reaction of hemagglutination inhibition (**Lutsyk et al. 1980**). The following carbohydrates and glycoproteins were used for this purpose: D-galactose, α-D-glucose, D-xylose, furanose (Institute of Chemistry, Czech Republic), N-acetyl-D-galactosamine, D-galactosamine chloride, D-glucosamine chloride, D-mannose (Chemapol, Czech Republic), L-ramnose, L-ribose, D-fructose (Pharmacia, Switzerland), L-arabinose, glucoso-6-phosphoric acid, phosphogluconic acid, fructoso-1,6-biphosphate (Reanal, Hungary), 2-deoxy-D-glucose, lactose, maltose, α-methyl-D-mannoside, β-D-thioglucose, methyl-α-D-glucopiranoside (Serva, Germany), D-galacturonic acid, D-glucuronic acid, dulcite, inositol, D-mannitol, sorbitol (Institute of Bioorganic Chemistry, RAS, Russia).
The degree of HAR inhibition was expressed as a minimal dose of carbohydrate, required for complete inhibition of HAR, and defined as the last dilution, when the inhibition of the reaction of hemagglutination of erythrocytes is still observed.
To study the impact of carbohydrates on bacteria adhesion to the cucumber roots, the cells were precipitated by centrifugation (OPN-8, Bishkek, Kirgiziya) at 5.000 g for 15 min, washed with the physiological solution three times, then resuspended in the latter making the optic density of the suspension up to 0.4 units. Then it was titrated in the wells by the addition of 0.05 ml of bacterial suspension to the dilutions 0.05 ml of 0.3 M solution of D-glucosamine chloride and inculbated under ambient conditions for 1 h. The cells were precipitated in the above-mentioned conditions and washed with the physiological solution; the optic density of the prepared suspension was 0.4 units. Then 0.1 g of cucumber roots was added 2 mL of the obtained suspension of cells and left for 1 h at room temperature. The roots were washed thrice with the physiological solution to remove the non-adhered cells, triturated in the mortar with 10 ml of the physiological solution and plated as dilution series on agarised potato medium. The number of colonies was counted with their subsequent calculation per 1 g of wet roots. In the control variant of experiments the cells of bacilli were treated with the physiological solution. In one experimental variant the cells of bacteria were not washed off the carbohydrate after the interaction with the latter.

Statistical analysis

Microsoft excel (Microsoft Corporation, USA) was used to analyze data on the average of three replicates (±SE) obtained from three independent experiments. Differences were compared for statistical significance at the P-level less than 0.05 ($P < 0.05$) (**Lakin 1990**).

Results and Discussion

The adhesion of bacteria is known to depend on many factors, including the stage of culture development (**Kurdish et al. 1998**). We have demonstrated that the cells of *B. subtilis*, cultivated for 24 h, adhered to the surface of glass granules in the maximal amount in the exponential growth phase. In the latter case $(1.24±0.06)·10^8$ cells were sorbed to 1g of glass. The bacteria, cultivated for 48 and 72 h, adhered to glass in a lesser amount. Their number on the glass amounted to $(0.55±0.03)·10^8$ and $(0.49±0.03)·10^8$ cells/ 1g of glass, respectively (Fig. 1).

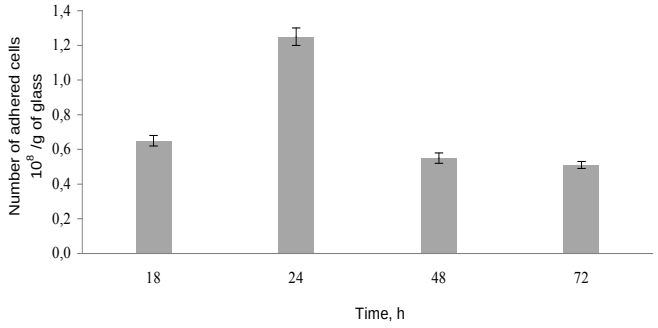

Figure 1 The adhesion of *Bacillus subtilis* IMV B-7023 to glass particles depending on the time of cultivation

The results required explanation of the differences in the adhesion to the glass surface of bacteria cultivated during different time periods. to the glass surface.

An important factor defining the efficiency of bacteria adhesion to solid materials, is known to be the hydrophobicity of interacting surfaces (**Kurdish 2010; Kurdish et al. 1998; Fletcher 1979**). We have studied the dependence of the surface hydrophobicity of *B. subtilis* IMV B-7023, on the phase of development. During the cultivation period of 18 h this index amounted only to 7 % (Fig. 2).

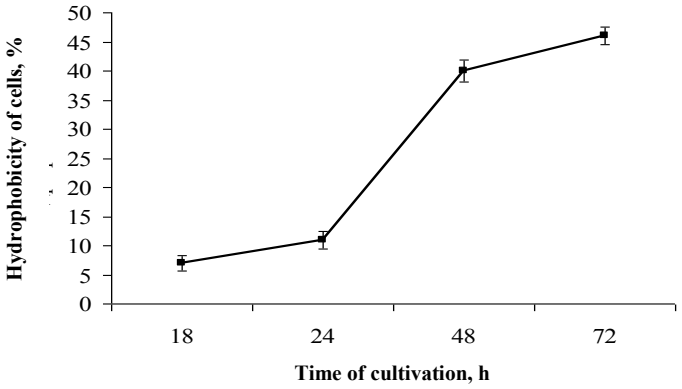

Figure 2 The dependence of hydrophobicity of *Bacillus subtilis* IMV B-7023 on the duration of cultivation

With the prolongation of the duration of bacilli cultivation to 24 h the hydrophobicity of cells increased up to 11 %, and after 48 and 72 h of cultivation – up to 40 and 46 %, respectively. Therefore, regardless of the significant role of hydrophobic interaction in bacteria adhesion **(Fletcher 1979; Kurdish *et al.* 1998)**, this factor did not play the decisive role in the maximal adhesion of *B. subtilis* to glass particles after 24 h of the cultivation.
Our further experiments have shown that a considerable impact on the adhesion process is made by the motility of cells **(Kurdish 2010)**. The microscopy of bacteria suspension samples, taken after 24 h of cultivation, testifies to the fact that all the cells of the population are mobile.
At the same time the majority of bacteria, cultivated for 72 h, were immobile and had the form of spores. Therefore, an important factor, determining the adhesion of bacteria to the abiotic surface of glass, is the motility of *B. subtilis* cells. The surfaces of bacterial cells and glass are known for their negative charge. During their interaction there are repulsive forces, decreasing the adhesive properties of bacilli. However, the motility of cells allows bacteria to overcome the repulsive forces, occurring between these surfaces, and to adhere to negatively charged surfaces, including the glass particles (**Fletcher 1979; Kurdish et al. 1998; Gordienko et al. 2009).**
Quite a different principle was observed while studying the adhesion of *B. subtilis* IMV B-7023 to the cucumber roots. Here the extension of the cultivation period resulted in the improvement of adhesive properties of the studied cells until the stationary phase of growth 48 h. Thus, after the 60 minute interaction of the cucumber roots and the bacteria, cultivated for 24 - 48 h, the number of bacilli adhered to their surface was $0.88 – 2.3·10^6$ cells/g (Fig. 3). When *B. subtilis* were cultivated longer (72 h), only $0.35·10^6$ CFU/g adhered to the cucumber roots which came up to only 15.2 % from the number of adhered cells selected from the stationary phase of growth (48 h).

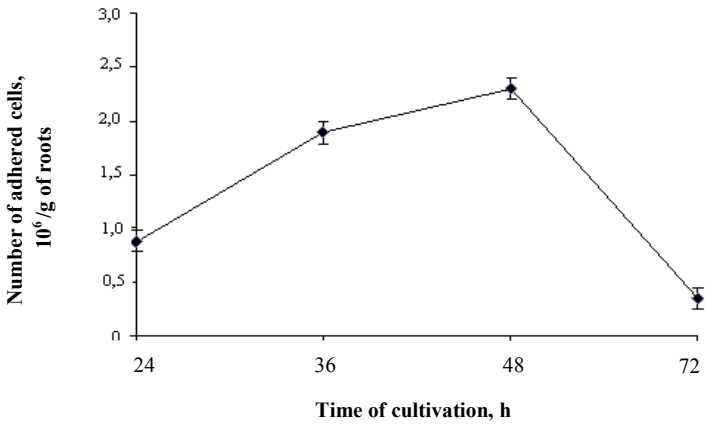

Figure 3 The adhesion of *Bacillus subtilis* IMV B-7023 to the cucumber roots depending on the duration of bacteria cultivation

These differences in the adhesion of bacilli to the cucumber roots were possibly caused by the specific interaction between *B. subtilis* and the root surface of these plants. There were found the lectins localized on the surface of phosphate-mobilizing bacteria *B. subtilis* IMV B-7023. The mentioned lectins are known for certain carbohydrate specificity.

The most vivid manifestation of the lectin activity regarding the trypsinized rabbit erythrocytes was observed for *B. subtilis* IMV B-7023 after 48 h of cultivation. The reaction of inhibiting the hemagglutination of rabbit erythrocytes with *B. subtilis* IMV B-7023 bacterial cells demonstrated that out of 30 carbohydrates tested in this study the carbohydrate specificities of surface lectins were observed only regarding D-glucosamine chloride (0.05 M), D-galacturonic (0.1 M) and D-glucuronic acids (0.00625 M).

The highest affinity of surface lectins of *B. subtilis* IMV B-7023 was demonstrated to D-glucosamine chloride. The interaction between bacterial cells and the carbohydrate, towards which the specificity of the lectins of bacilli was revealed, diminished the adhesion of cells to the cucumber roots considerably. Not treated with the carbohydrate $9.3 \cdot 10^6$ bacterial cells sorbed per 1 g of roots was taken as 100 % control. In the case D-glucosamine chloride treatment the adhesion of bacilli to the cucumber roots decreased by 26 % being $6.9 \cdot 10^6$ CFU/g of roots (Fig. 4).

The obtained results testifed to the great importance of the surface lectins for the adhesion of *Bacillus subtilis* IMV B-7023 to the cucumber roots. It is noteworthy that the representatives of *Bacillus* genus along with the surface lectins are also capable of synthesizing extracellular lectins **(Podgorskii et al. 2014)**.

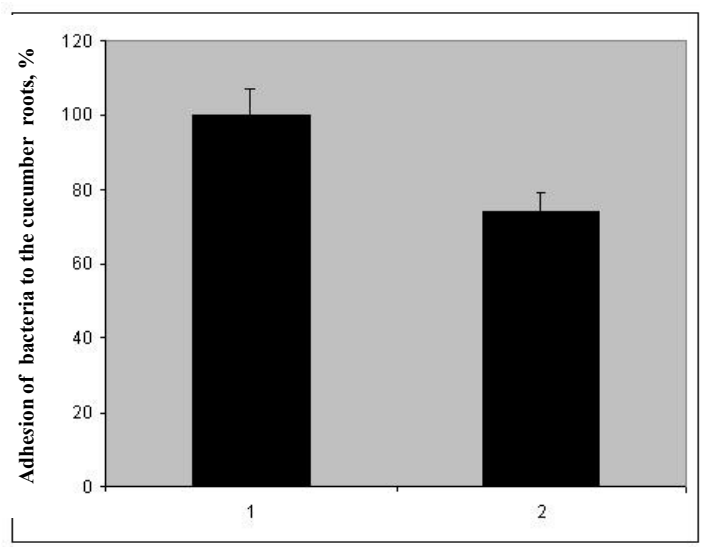

Figure 4 The impact of lectin-carbohydrate interaction on the adhesion of *Bacillus subtilis* IMV B-7023 to the cucumber roots, Konkurent cultivar. The adhesion of bacteria in the control (1) and after their treatment with D-glucosamine chloride (2).

It has recently been shown that many bacteria use carbohydrate-specific lectins which are expressed as part of capillary protein appendages expanding from their surface called fimbrie or pili for the adhesion to biotic surfaces **(Mirja Hartmann and Thisbe Lindhourst 2011)**. A relevant role of the surface lectins was established for the adhesion of *Azospirillum brasilense* Sp1 to wheat roots and for the formation of defensive reactions of plants **(Nikitina 2001)**. For instance, it was demonstrated that the treatment of wheat seeds with the solution containing 40 µg ml^{-1} of *Azospirillum brasilense* Sp1 lectin lead to the synthesis of nitrogen oxide in the roots of sprouts, which is one of the inducers of the processes of adaptation of these plants to stress factors **(Alen'kina, Nikitina 2010)**.

Therefore, the surface lectins of *B. subtilis* IMV B-7023 plaied a significant role in the adhesion of bacteria to the biotic surfaces (cucumber roots). They played the role of adhesins in this process. At the same time when these bacteria interacted with abiotic surfaces, the leading role was played by the motility of cells helping to overcome the repulsive forces between negatively charged surfaces of bacteria and glass particles and ensure the adhesion of cells to a solid surface.

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ISOLATION, PURIFICATION AND CHARACTERIZATION OF A BACTERIOCIN WITH BROAD SPECTRUM ACTIVITY FROM *Lactococcus lactis* JC10 FROM PERISHABLE PAPAYA FRUIT

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ABSTRACT

Food-grade bacteria capable of producing bacteriocin with preservation abilities have been isolated from perishable papaya. Characterization of the bacteria isolated on MRS agar, by using 16S rRNA sequence analysis, exhibited antagonistic effect towards the growth of a wide range of Gram + and Gram - bacteria including pathogens also. Extracellularly produced bacteriocin was purified by ion-exchange chromatography on a Fast Protein Liquid Chromatography (FPLC) system equipped with a Mono-Q column. The purity rate and molecular mass of 21 kDa of this compound were determined using SDS-PAGE. Activity units (AU) of bacteriocin were increased in each step of purification, reaching up to 9500 AU/mL. The increase in the activity units directly affected the antimicrobial activity of purified bacteriocin, resulting in an increase of the inhibition zones against indicator bacteria. It withstood very high temperature, up to 115.6 °C, for 10 min but lost activity after autoclaving (121°C), wider pH range, from 3.0 to 10.0, complete inactivation in the presence of proteolytic enzymes (protease and α chymotrypsin) and storage stability up to 6 months.

Keywords: Bacteriocin, *Lactococcus lactis* JC10, purification, biopreservative, antimicrobial activity

INTRODUCTION

The ability of Lactic Acid Bacteria (LAB) to inhibit the growth of other bacteria has been known for many years. LAB have been defined as "generally recognized as safe" (GRAS) microorganisms .The inhibitory activity of LAB may be attributed to their acidification of the medium, competition for substrates and the production of antimicrobial compounds such as Hydrogen peroxide, Diacetyl and antimicrobial peptides known as Bacteriocins (**Parente *et al.*, 1999**).

Bacteriocin is one of the antagonistic compounds found to posses major applications in food industries as safe food preservative. The bacteriocins produced by LAB have been classified into four groups: class I, small, heat-stable, lanthionine-containing peptides (≤ 5 KDa); class II, small, heat-stable, lanthionine-containing peptides (≤ 10 KDa); class III, large, heat-labile proteins (≥ 30 KDa); and class IV, complex proteins composed of one or more chemical moieties, either lipid or carbohydrate. Class I and class II bacteriocins are the most likely candidates for use as biopreservatives in the food industry. Now a days, food safety is an important issue of international concern. Consumers of this decade are interested in food without harmful chemical preservatives and with additional health benefits. Packaged food items available in market contain variety of chemical preservatives that may alter chemical constituents, Nutritional as well as organoleptic qualities of food thereby causing serious adverse health effects (**Messi *et al.*, 2003**). Thus biopreservation of food has emerged as an attractive and safe approach. Among biopreservatives, bacteriocins have received increased attention especially due to their low toxicity, as they are degradable by digestive enzymes (**Cleveland *et al.*,2001).** Formulations containing bacteriocins such as Nisaplin (Nisin) and ALTA 2351(Pediocin PA-1) have been added to food to increase shelf life and increase food safety (**Mills *et al.*, 2011**).

Many researchers suggested that useful criteria for antagonistic activity of bacteriocins as: 1) the presence of an essential, biologically active protein or peptide moiety, 2) inhibitory activity against closely related bacterial species and 3) a bacteriocin's mode of action .

In an extensive suvey of bacteriocin producers, it was observed that about 43% (out of 162 strains) Lactococcal strains tested were capable of producing bacteriocin. On the other hand Nisin is the only bacteriocin from *Lactococcus lactis* that has been studied in detail. The inhibitory spectra of the different lactococcal bacteriocins vary but they are generally narrower than that of Nisin (**Geis *et al.*,1983**). Schnell et al 1988 stated that many of the Lactococcal bacteriocins described are very different from Nisin and does not belong to the lantibiotic family of bacteriocins.

The objective of this present study was to describe a novel bacteriocin produced by *Lactococcus lactis* JC10 isolated in our laboratory from papaya fruit. This bacteriocin having potential antibacterial activity including pathogens, thermal stability and small production time can be used in food industry as an alternative for chemical preservatives. Further, purification and characterization of this bacteriocin was studied in detail for their potential application as food preservative in future.

MATERIAL AND METHODS

Bacterial strains and culture conditions

The following food spoilage bacterial strains were employed in the screening for potent bacteriocinogenic strain: *Lactobacillus plantarum* 2083, *Lactobacillus plantarum* 2592, *Lactobacillus casei* 2737 (Collected from NCIM, Pune, India). MRS medium (Hi-Media, India) was used for sub culturing and strain maintenance for these three indicator strains. These strains were maintained by sub culture aerobically at static condition (37°C for 24 hrs).

The following spoilage and pathogenic bacterial strains were considered in the screening for antagonistic activity : *E.coli* NCIM 2065, *Pseudomonas putida* NCIM 2650, *Klebsiella pneumoniae* NCIM 2707, , *Bacillus megaterium* NCIM 2034, *Bacillus subtilis* NCIM 2545, *Vibrio fischeri* NCIM 5269, *Enterobacter aerogens* NCIM 2340, *Micrococcus luteus* NCIM 2169, *Staphylococcus aureus* NCIM 2127, *Proteus vulgaris* NCIM 2027, *Alcaligenes viscosus* NCIM 2446, *Leuconostoc mesenteroides* NCIM 2073 (collected from NCIM, Pune), *Salmonella typhi* MTCC3216, *Shigella flexneri* MTCC1457, *Bacillus thuringiensis MTCC6941* (collected from MTCC, Chandigarh).All these strains were maintained in Nutrient medium (Hi-Media, India). Strains were stored as lyophilized frozen stocks at -20 °C when strains are not in regular use.

Screening and isolation of potent lactic acid bacteria

Food samples (acidic) (158) were collected from local market in sterilized zip-lock packet. A little fraction of each sample was vortexed in sterilized distilled water (5 ml) in a tube. A fixed amount of sample and indicator culture were mixed with molten soft agar medium and spread on MRS hard agar plate. Then

the plates were incubated overnight at incubator at 37°C for a selecting appearance of producer colony of LAB with a hollow zone around it. The producer colony was randomly picked and purified by streaking onto MRS agar plate. The purified colonies were primarily identified by Gram staining and catalase tests. Only gram positive, catalase- negative colonies were presumed to be LAB. The pure single colony was inoculated in MRS broth overnight at 37°C for bacteriocin production. 1.5 ml of broth culture was centrifuged at 10,000 rpm for 10 minutes (at 4°C). Cell free supernatant (CFS) was collected in a fresh sterilized eppendorf. Crude CFS was adjusted at pH 7.0 by 1 (N) NaOH (Merck, Germany) to rule out acid inhibition. Inhibitory activity from hydrogen peroxide was ruled out by the addition of catalase (250 IU/ml). In the meantime indicator cultures were mixed with molten MRS soft agar medium and spread on MRS hard agar plates and kept for solidification for approximately 15 minutes. 5 µl neutralized CFS was tested on different indicator cultures. Plates were incubated for 24 hrs, at 37°C to monitor any kind of inhibition zone against all the indicator cultures. Degree of antagonism was determined by measuring the inhibition zone diameter against respective indicator culture using an agar well diffusion assay.

Characterization of the antimicrobial compound

Chemical nature of the antimicrobial compound was determined by application of several proteolytic enzymes (Pepsin, Trypsin, Proteinase K, Protease, α-chymotrypsin) on cell free supernatant. 1 mg/ml stock of each proteolytic enzyme was mixed with crude CFS in 1:1 (v/v) ratio and incubated for 30 minutes at 37°C. With treated CFS, spot on lawn assay was performed on indicator culture *Lactobacillus plantarum* 2083. Results were observed on the next day to determine the chemical characteristics of the antimicrobial compound. Each and every experiment was done in triplet with proper control related with the experiment.

Characterization and identification of isolated strain

Morphological study

Morphology was determined by gram staining procedure initially. The strain isolated was inoculated into sugar broth tubes to find out the fermentation capability of the organism in different sugars. Catalase activity was tested by spotting colonies with 3% Hydrogen peroxide.
The isolated viable bacterium was examined with scanning electron microscopy (SEM) later on. Isolated LAB strain used in scanning electron microscopy study was obtained from 24 hrs old culture in MRS media (37°C). The O.D was adjusted to 0.1 and 1.5 ml of cell suspension was taken in sterilized eppendorf and centrifuged at 6000 rpm for 10 minutes. Supernatant was discarded, cell pellet was dissolved in 0.1 M phosphate buffer (pH 6.9) and washed twice with 0.1 M phosphate buffer and stored at 4°C. After centrifuging the eppendorf (6000 rpm, 10 minutes), cell pellet was fixed with 5% (v/v) glutaraldehyde (EM grade, Merck, Germany) for 2 hours at 4°C. Fixative was prepared in sterilized sodium cacodylate buffer (pH 7.4). The fixed pellet was dehydrated in a graded ethanol series (30% to 100%), incubating in each grade for 10 minutes. The dried preparation was coated with gold/palladium (60:40) with thickness below 10 nm using deposit thickness monitor. It was then observed under electron microscope (FEI Quanta- 200 MK2) with a magnification of 12000X.

16s rRNA sequence analysis

The strain was identified according to Sneath et al. (**Sneath *et al*.,1986**) and Thomas et al. (**Thomas *et al*., 1980**). Preliminary identification was based on its morphological and biochemical characteristics in addition to its carbohydrate fermentation profile following Bergey's Manual of Systematic Bacteriology (**Garrity *et al*., 2005**). Finally it was characterized according to its 16S rDNA sequence analysis. The 16S rRNA gene of the strain *Lactococcus lactis* JC10 was amplified by the method described earlier (Das et al, 1996). Primers used for the amplification of 16S rRNA were 5'-GAG TTT GAT CCT GGC TCA G-3' (forward primer) and 5'-AGA AAG GAG GTG ATC CAG CC-3' (reverse primer). Genomic DNA amplification was performed with a Thermal Cycler, Model PCT-200 (M.J. Research, Waltham, MA, USA) with the following temperature conditions: initial denaturation step at 94°C for 4 min; followed by 30 cycles of 62°C for 1 min, 72°C for 1.5 min and 94°C for 1 min and final extension at 72°C for 7 min. The PCR product was purified by using the QIAquick PCR purification kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Gel-purified 16S rRNA was sequenced using a CEQ Dye terminator cycle sequencing kit in an automated DNA sequencer Model CEQ 8000 (Beckman Coulter, Fullerton, CA, USA) (**Panday *et al*., 2010**). Nucleotide sequences thus obtained were assembled using the sequence alignment editor program Bioedit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Subsequently these sequences were blasted against the GenBank database (http://ncbi.nlm.nih.gov/BLAST) (Altschul et al, 1997). Evolutionary distance to other strains of *Lactococcus* was computed by neighbor joining method (**Saitou *et al*. 1987**). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and phylogenetic tree was constructed by software MEGA5 (**Tamura *et al*., 2007**).

Effect of thermal treatments on bacteriocin activity

Thermal stability test was performed with the neutralized CFS of the isolated LAB strain. The effect of temperature on stability of bacteriocin was determined in the CFS subjected to treatment at 100°C for 1 hr, 100°C for 2 hrs, 100°C for 2 hrs, 108°C for 10min, 115.6°C for 20 min and 121°C for 15 min. Immediately after each treatment, samples were cooled under refrigeration and residual activity was determined by spot on lawn assay on indicator strain *Lactobacillus plantarum* 2083. A positive control, freshly produced neutralized CFS was tested in parallel.
The effect of extended storage at low temperature (-20°C and 0°C) on bacteriocin stability was also determined by placing CFS in a deep freeze for 270 days. Residual activity was determined by the spot on lawn assay, as previously described. In all instances, a positive control, consisting of freshly prepared neutralized CFS was tested in parallel.

Effect of pH on bacteriocin activity

About 5ml aliquot of purified bacteriocin was taken in test tubes and pH values of the contents were adjusted from 2 to 10 individually using either dilute NaOH or HCl (1M NaOH or 1M HCl solution). After allowing the samples to stand at room temperature for 2 hours, antimicrobial activity was assayed.

Effect of organic solvent on bacteriocin activity

To study the effect of different solvents on bacteriocin, neutralized CFS was mixed with 1:1 (v/v) Ethanol, Isopropanol, Acetone, Methanol, Butanol. It was kept at room temperature for 30 min. After evaporation of solvents, 5 µl of solvent treated CFS was spotted on lawn of indicator organisms with appropriate control. Activity of the antimicrobial compound was similarly evaluated in presence of β mercaptoethanol.

Antimicrobial spectrum of the bacteriocin

Neutralized CFS was tested against several gm+ and gm- bacteria. Activity of bacteriocin was tested by constructing soft agar lawn of each test microorganism on MRS plate separately and spot of neutralized CFS (5 µl) was given. Each plate was incubated (37°C, 24 hrs) and examined for inhibition zone to be appeared on the next day.

Extraction of crude bacteriocin

Two liter culture of *L. lactis* JC10 was grown in MRS broth media (pH 6.8) at 37 °C at static condition for 5 hours. Cell free supernatant obtained after centrifuging the broth culture at 10000 rpm for 15 min at 4 °C was used as crude bacteriocin.

Concentrating bacteriocin sample

Diluted solution of crude bacteriocin was concentrated 10 times by lyophilization at 4°C.

Ammonium sulfate precipitation

Concentrated crude bacteriocin was subjected to ammonium sulfate fractionation. Ammonium sulfate was gently added to the supernatant maintained at 4 °C to obtain 40% saturation, and the mixture was stirred for 4 h at 4 °C. It was then centrifuged for 30 min at 20000×g at 4 °C; the resulting pellet was resuspended in required amount of 0.1 M phosphate buffer (pH 6.9). It was considered as partially purified bacteriocin.

Determination of protein content

Due to the presence of tween 80 in MRS medium, protein determination by Lowry method gave a false estimation of protein content in the medium. To avoid interference of detergent, protein content of medium was determined by modified Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) (**Lowry *et al*., 1951**) using Bio-Rad RCDC protein assay kit (detergent compatible) as per manufacturer instruction. Bovine serum albumin was used as a standard. Specific activity was defined as the ratio of bacteriocin activity (AU/mL) to protein concentration (mg/mL).

Bacteriocin production and purification

The supernatant fluid of a 5 hr *Lactococcus lactis* JC10 culture was collected by centrifugation at 10000 rpm for 10 minute and filtered through a membrane filter (0.45 µm pore size, Millipore). The proteins were precipitated with 40%

ammonium sulphate overnight at 4°C. The precipitate was recovered and exhaustively dialyzed against phosphate buffer 0.1M pH 6.9 which was crude bacteriocin. This was further purified by FPLC anion exchange chromatography. The sample was absorbed on a Mono Q 5/50 GL (AKTAFPLC, GE Life Sciences) column, previously equilibrated with 20 mM Tris-HCl, pH 8.0 (buffer A). Elution was accomplished with an increasing concentration of 20mM Tris-HCl, pH 8.0 + 1 M NaCl (buffer B). Fractions were collected and assayed for antimicrobial activity.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)

The antimicrobial preparations and FPLC purified sample were examined on 10% SDS-PAGE (**Laemmli, 1970**). Samples and molecular weight standards 1mg/ml were dissolved in sample buffer and loaded onto the gel. After electrophoresis at 40 milliamps for approximately 4 hours, the gel was stained with coomassie brilliant blue dye.

RESULTS

Identification of isolated strain

Microbial characteristics of *Lactococcus lactis* JC10 was found as gram-positive,spherical shaped, non-endospore forming, catalase negative bacterium (Tab 1). It fermented a wide array of sugars. It could utilize glucose, sucrose, mannitol, lactose etc producing organic acid only but no CO_2 gas. Acid production was maximum in the fermentative degradation of lactose. Methyl red test, V-P test, Starch degradation test, Citrate production test and Gelatin liquefaction test showed negative results.

Table 1 Morphological and biochemical characteristics of *Lactococcus lactis* JC10

Morphological		**Biochemical**	
Size & shape	Spherical	Test	Result
Arrangements	Discrete	Glucose fermentation	A++, G-
Gram reaction	Positive	Sucrose fermentation	A++, G-
Endospore formation	No	Mannitol fermentation	A++, G-
		Lactose fermentation	A+++, G-
		Methyl red test	_
		V-P test	_
		Starch degradation	_
		Citrate production	_
		Gelatin liquefaction	_
		Indole test	_
		Catalase test	_

Legend: A- acid,G -gas

Morphologically cells structure was evidenced from SEM image (Figure 1). The cells are found to be spherical or ovoid shape, within the size range of 1.2 µm to 1.5µm , occuring in pairs or short chains.

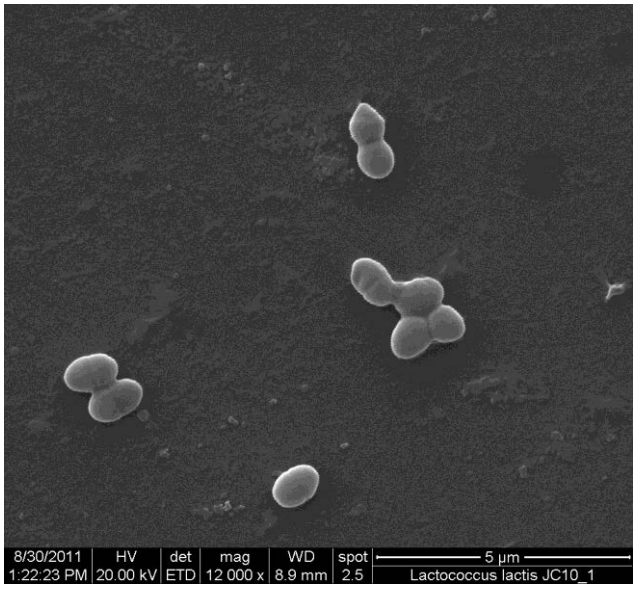

Figure 1 Electron micrograph of bacteriocin producing *Lactococcus lactis* JC10

The result of the biochemical identification was further confirmed by amplification of the 16s rRNA genes. PCR amplification and sequencing of its 16s rDNA gene was edited to a total length of 1438 bp. BLAST search showed sequence homology with *Lactococcus lactis*. Phylogenetic tree based on different species of *Lactococcus* was constructed using neighbor joining method (Figure 2).

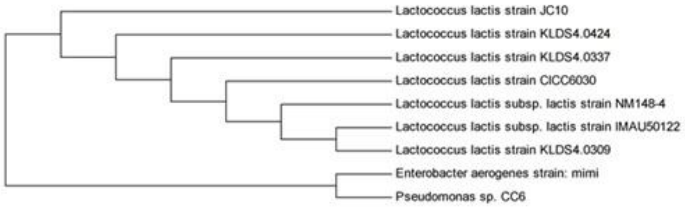

Figure 2 Phylogenetic relationship of 16s rRNA sequence of *Lactococcus lactis* JC10 with other reference strains of *Lactococcus*.

Result indicated that the strain JC10 was very closely (greater than 97% similarity) related with *Lactococcus lactis* strain. It is known that sequence similarity >97% is acceptable level for microbial identification and the microbial strain shall be considered as same species (**Janda *et al*., 2007**). Based on information from phenotypical, physiological and molecular testing, the strain was identified as *Lactococcus lactis*. Further based on microscopic and biochemical techniques this LAB stain was named as *Lactococcus lactis* JC10.

Bacteriocin purification and molecular weight determination

The supernatant fluid of a 5 hr *Lactococcus lactis* JC10 culture was collected by centrifugation at 10000 rpm for 10 minute and filtered through a membrane filter (0.45 µm pore size, Millipore). The proteins were precipitated with 40% ammonium sulphate overnight at 4°C (**Keen, 1966**). The precipitate was recovered and exhaustively dialyzed against deionized water and this was crude bacteriocin. The dialysate formed an insoluble portion which contained most of the antimicrobial activity (**Joerger *et al*., 1986**). This was purified by FPLC anion exchange chromatography (Figure 3).

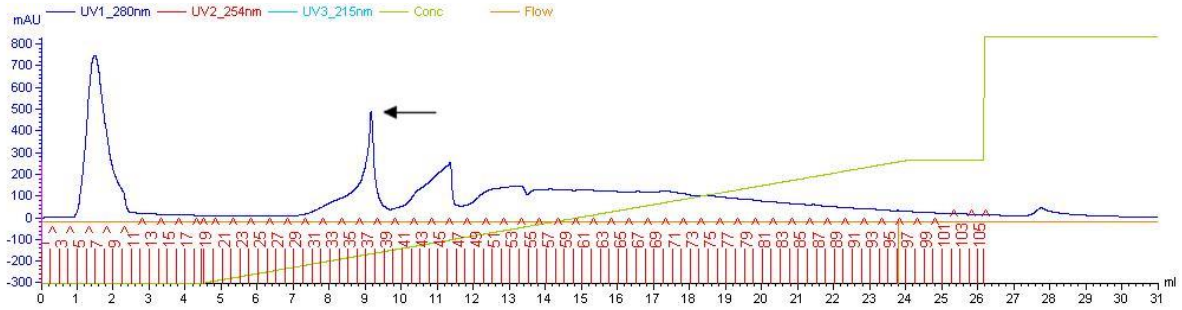

Figure 3 Fast Protein Liquid Chromatogram of purified bacteriocin produced from *Lactococcus lactis* JC10. Fractions (36-38) showed highest bacteriocin activity is indicated by arrow.

The sample was absorbed on a Mono Q 5/50 GL (AKTA FPLC, GE Life Sciences) column, previously equilibrated with 20 mM Tris-HCl, pH 8.0 (buffer A). Elution was accomplished with an increasing concentration of 20mM Tris-HCl, pH 8.0 + 1 M NaCl (buffer B). Fractions were collected and assayed for antimicrobial activity. Pooled fractions from 34 to 38 revealed the highest bacteriocin activity which yielded (at recovery of 22.07% and a 3.75 fold purification as indicated in (Tab 2)) a titre of 9500 AU/ml and a specific activity of 2794 AU/mg protein. The results of the purification procedure were summarized in Table 2.

Table 2 Purification of bacteriocin from *Lactococcus lactis* JC10

Purification stage	Volume (ml)	Activity (AU/ml)	Total activity (10^4)	Protein (mg/ml)	Specific activity (AU/mg)	Purification factor	Recovery (%)
Culture supernatant	500	6500	325	15.4	422	1	100
Ammonium sulfate precipitation (0-40%)	20	7600	15.2	10.2	745	1.76	66.23
Purified bacteriocin	10	9500	9.5	3.4	2794	3.75	22.07

SDS-PAGE analysis of the bacteriocin obtained by FPLC revealed a single band with a molecular mass of approximately 21 kD (Figure 4) . The bacteriocins of lactic acid bacteria belonging to class III have molecular weight >10 KD . So the higher molecular mass of bacteriocin of *Lactococcus lactis* JC10 (21KD) suggested that it might belong to class III bacteriocin group.

FPLC purified bacteriocin showed larger zone of inhibition than control (Figure5) indicating a 3.75 fold purification.

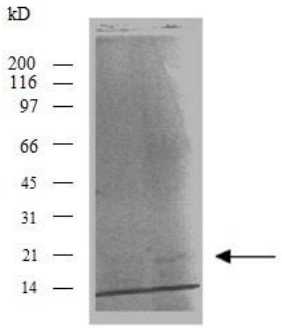

Figure 4 SDS PAGE of FPLC fraction. FPLC pufified fraction showing zone of inhibition on MRS plate in SDS PAGE shows 21 kD band indicated by arrow.

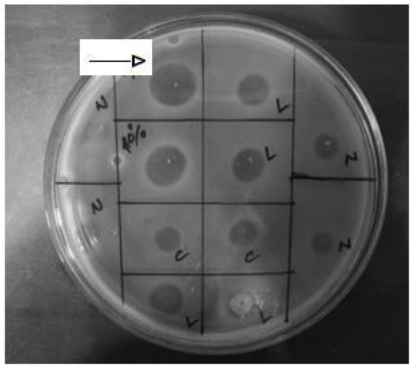

Figure 5 Zone of inhibition of bacteriocin. FPLC purified fraction is marked by arrow.

Antimicrobial spectrum

The antimicrobial activity of bacteriocin was tested against a number of Gram + and Gram - bacteria including pathogens (Tab 3). Table 3 shows sensitivity of the various strains to the bacteriocin produced by *Lactococcus lactis* JC10 as measured with the Spot-on-lawn method.

Table 3 Antimicrobial spectrum of bacteriocin produced by *Lactococcus lactis* JC10.

Name of microorganisms	NCIM/MTCC No.	Zone of inhibition (mean of three trials) (mm)
E.coli	NCIM 2065	14
Pseudomonas putida	NCIM 2650	12
Klebsiella pneumoniae	NCIM 2707	12
Salmonella typhi	MTCC 3216	16
Shigella flexneri	MTCC 1457	14.5
Bacillus thuringiensis	MTCC 6941	12
Bacillus megaterium	NCIM 2034	12
Bacillus subtilis	NCIM 2545	14
Vibrio fischeri	NCIM 5269	13.5
Enterobacter aerogenes	NCIM 2340	15
Micrococcus luteus	NCIM 2169	12
Staphylococcus aureus	NCIM 2127	16
Proteus vulgaris	NCIM 2027	0
Alcaligenes viscosus	NCIM 2446	0
Leuconostoc mesenteroides	NCIM 2073	11.5
Lactobacillus plantarum	MTCC 2083	13
Lactobacillus plantarum	MTCC 2592	14
Lactobacillus casei	MTCC 2737	13

The bacteriocin obtained from *L. lactis* JC10 showed very strong inhibitory activity against *E.coli, Shigella flexneri, Enterobacter aerogenes* etc which are Gram negative bacteria. It is known that generally bacteriocins of lactic acid bacteria do not inhibit the growth of Gram Negative bacteria. This unusal result indicates that this bacteriocin has a broad antimicrobial spectrum of activity.

It was not active against *Proteus vulgaris* and *Alcaligenes viscosus*.

The largest zone of inhibition(16 mm) was obtained against *Staphylococcus aureus* NCIM 2127 and *Salmonella typhi* MTCC 3216. This bacteriocin is reported to be active against other pathogens also.

Effect of temperature, solvents, enzymes and pH on bacteriocin activity

Bactericin from *Lactococcus lactis* JC10 has shown remarkable temperature stability. Figure 6 shows the effect of temperature on bacteriocin activity in terms of inhibition zones. It has been found to be thermostable in nature. It was equally stable in wide range of temperatures ranging from -20°C to 115.6°C as compared with control (Figure 6).

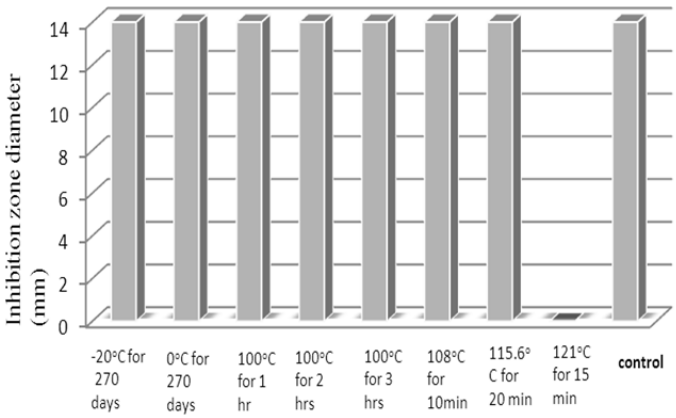

Figure 6 Thermal stability of bacteriocin

It was stable for more than 6 months in refrigerated condition. Full stability was recorded after autoclaving the bacteriocin at 10 lb pressure for 20 min (115.6 °C). But it lost its activity after autoclaving at 15 lb pressure for 15 min (121°C).
This Bacteriocin was found to be stable in presence of different organic solvents (Tab 4).

Table 4 Effect of different solvents on bacteriocin produced by *Lactococcus lactis* JC10

Organic chemicals	Inhibitory zone diameter on MRS plate (mm)
Ethanol	11
Isopropanol	10.5
Acetone	11
Methanol	11
Butanol	10
2 mercaptoethanol	0

Restoration of complete function of bacteriocin in presence of several organic solvents broadens its application in several natural environments. The antimicrobial activity of the compound was totally lost in presence of 2 mercaptoethanol.
Treatment with proteolytic enzymes like protease and α-chymotrypsin nullify the antimicrobial property of neutralized CFS showing no inhibition zone on Spot-on-lawn assay whereas catalase, pepsin, trypsin and proteinase K showed zone of inhibitions of varied diameter showing that the activity was not affected by these enzymes (Tab 5).

Table 5 Effect of different enzymes on purified bacteriocin

Enzymes added	Inhibitory zone diameter on MRS plate (mm)	Activity (AU/mL)
Catalase (Sigma No. C9322)	12	3200
Pepsin (Sigma No.P6887)	11	3200
Trypsin (Sigma No. T6567)	12	3200
Proteinase K (Sigma No.P2308)	10	3200
Protease (Sigma No.P2714)	0	-
α-chymotrypsin (Sigma No.C4129)	0	-

This protease sensitivity assay demonstrated that this antimicrobial substance was proteinaceous in nature.
The residual activities of the purified bacteriocin from *Lactococcus lactis* JC10 revealed that the bacteriocin retained its total activity in the pH range of 3-10 with no change in AU/ml .This indicates that the bacteriocin is pH stable and it could retain its antimicrobial activity when there was a shift to acidic or basic range. Stability of bacteriocin at different pH range is a limiting factor for recommending its use in food items.

DISCUSSION

Within the last several years, studies of bacteriocin have attracted significant attention because of their potential use in many fields. Our research has focused on a search for new bacteriocin produced by generally recognized as safe (GRAS) bacterial strains. The results, presented here indicates that our isolate *Lactococcus lactis* JC10 have shown to produce maximum level of bacteriocin (6400 AU/ml) only within 5 hours of incubation. There is no such report of food grade LAB to produce highest level of bacteriocin within such short time span. This might be very useful in commercial bacteriocin production or generating a high yielding starter culture for manufacturing fermentative food products.
The bacteriocin studied was thermostable as heating at temperature higher than 100°C (115.6°C) did not destroy its inhibitory activity. Several studies have been reported that the bacteriocin treated at 100°C for 120 min and 121°C for 15 min were stable (**Do *et al*., 2001**; **Pilar *et al*., 2008**). Pediocin SJ-1 (**Schved *et al*.,1993)** was not affected by heat treatment for 30 min at 100°C. These examples clearly indicate that bacteriocins posses thermostable property. The heat stability of bacteriocin discussed here indicates that it could be used as biopreservative in combination with thermal processing to preserve the food products. Nevertheless, it exhibited activity at low temperatures and the protein maintains its initial activity i. e. same zone diameter of inhibition when it was kept at -20°C for six months. This noteworthy temperature stability (-20 °C to 115.6°C) of this bacteriocin make this bacteriocin more fitted candidate for commercialization. However more studies on these aspects are needed.
The inhibitory activity of this antagonistic substance was completely lost after treatment with either protease or α chymotrypsin, thereby revealing its proteinaceous nature. Other bacteriocins are also susceptible to these same enzymes.
The bacteriocin activity was totally lost by organic solvent 2-mercaptoethanol, which indicates that this bacteriocin might contain S-S linkage which is known to contribute to protein thermal stability. Although the reason for bacteriocin heat stability could be due to its complex nature.
Several protocols and chromatographic methods have been proposed for the analytical purification of bacteriocins. Chromatographic methods, such as ion-exchange or size-exclusion (gel filtration, are usually applied after an initial concentration step by salt precipitation (Ammonium sulphate precipitation) (**Muriana *et al.*, 1991**). This bacteriocin of interest was purified by a sequential concentration by lyophilization, salting out followed by ion exchange chromatography protocol using FPLC with Mono-Q column. The substance was bound to the Mono-Q matrix, indicating that the protein is anionic in nature. Because it is anionic, it is likely that its mode of action is different from class 1 and 2 bacteriocins, which are membrane active peptides and proteins. Further SDS PAGE analysis showed that the fraction showing antimicrobial activity was of high molecular mass (21 KD).
Though in general, bacteriocins posses narrow spectrum antimicrobial activity (**Riley *et al.*, 2002),** this bacteriocin from *Lactococcus lactis* JC10, on virtue of its broad spectrum antimicrobial activity, can be useful regarding biological food preservation. To get rid of hazards of chemical preservatives, bacteriocins could be employed.
Bacteriocins could also be utilized to develop new novel approaches for controlling food-borne bacterial disease-causing agents with extensively observed increased antibiotic resistance among all bacterial groups (**Gyles, 2008; Nathan *et al*., 2005**). Hence, application of purified bacteriocin (from *L.lactis* JC10) to preserve foods can be exercised. Apart from this, with respect to medical applications, isoloated LAB srain (*L.lactis* JC10) and bacteriocin produced by it, might play a role during in vivo interactions occurring in the human gastrointestinal tract contributing to gut health. Further research is needed to unravel the precise role of LAB and bacteriocins in this process.

CONCLUSION

The study revealed that bacteriocin from *Lactococcus lactis* JC10 isolated from papaya fruit posseses a wide spectrum of inhibitory activity against a number of pathogens. Bacteriocin with such thermostability and pH stability has a potential for application as a biopreservative in differenet thermally processed food products as such or in combination with other preservation methods. More studies are necessary to better describe the protein and elucidate its mode of action and genetic characteristics.

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DIVERSITY ASSESSMENT AND EPS PRODUCTION POTENTIAL OF CULTIVABLE BACTERIA FROM THE SAMPLES OF COASTAL SITE OF ALANG

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ABSTRACT

In this study diversity of cultivable bacteria and their exopolysaccharide production potential were investigated from the water and sediment samples of coastal site of ALang. The studied ten samples represented diversity in pH, conductivity, salinity and TDS, in the range of 7.25 to 8.92, 9.4 to 76.8 mS, 3570 to 35100 mg/L and 15120 to 53100 mg/L respectively. Total 141 bacteria were isolated from the collected ten samples, from which 105 were gram positive and 36 were gram negative in nature. Amongst the isolates 33% were able to produce variety of pigments. The diversity indices, including Shannon-Wiener index (H'), Richness, and Evenness Indices based on the metabolic characteristics of the organisms were calculated. The isolates were characterized morphologically and biochemically. Identification of 41 isolates was confirmed by 16S rRNA gene sequencing. They represented 22 genera and various species of these genera. The bacterial isolates were able to grow in the range of 3-25% NaCl concentrations. When 141 cultures were grown in liquid medium, the viscosity of the medium ranged between $9.20x10^{-6}$ to $2.09x10^{-4}$ m.pa.s^{-1}. The EPS yield in terms of dry weight also ranged from 0.76 to 10.7g/L.

Keywords: Alang, coastal, diversity indices, exopolysaccharide, phylogeny, pigment

INTRODUCTION

India has the coastline of 7516 kilometers, out of this Gujarat state contributes about 21% (~1600 kilometers) of the coastline **(Venkatraman, 2008; Parikh *et al*., 2012)**. The coastal region of the Bhavnagar is a center for varied economic activities like salt production, mining, fishing, ship building and breaking. Alang is the largest ship breaking yard in the world and is a census town of Bhavnagar district. Its economical importance with regards to its salt production value and ship breaking process makes it a valuable site for the microbiological studies. Studied saline sites of India are Sambhar salt lake, Rajasthan (**Sahay *et al*., 2012)**, Coastal regions of Gujarat, **(Dave and Desai, 2006)**, Little Rann of Kutchh, Gujarat **(Thomas *et al*., 2012)**, Tamilnadu, Andhra Pradesh **(Kumari *et al*., 2013)**, Maharashtra **(Deshmukh *et al*., 2011)**, Orissa **(Bal *et al*., 2009)**, and West Bengal **(Das *et al*., 2012)**. Some of the coastlines studied worldwide are the Great Salt Lake (Utah, USA), the Dead Sea (Israel), the alkaline brines of Wadi Natrun (Egypt), and Lake Magadi (Kenya) **(Nanjani and Soni, 2012)**. These habitats represent extreme conditions of high salinity, high pH, low oxygen conditions and different range of temperature values and are inhabited by halotolerant as well as halophilic microorganisms **(Moreno *et al*., 2013)**. Members of the domain bacteria are of special interest to scientists, as they play an important role in saline as well as hypersaline environments and have the potential to produce compounds of industrial interest, one of these compounds is the exopolysaccharide (EPS) **(Antoin *et al*., 2000; Hedi *et al*., 2014)**. Novel EPS with better characteristics can be developed from the isolates of such ecosystem than those of the existing one **(Bejar *et al*., 1998)**. EPS produced by microorganisms has a number of applications in pharma, food, petroleum and other industries **(Sutherland, 1990)**. Because of their unique properties they can be used as coagulants, thickening agents, binder, emulsifiers, stabilizers, lubricants and gelling agents **(Sutherland, 1997)**. EPS have been reported to increase the viscosity of solution at low pH values, as a good surface active agent for heavy metal remediation and provides gluing properties in soil aggregation **(Kalpan *et al*., 1987; Nisha *et al*., 2007)**. The saline site of Alang, Bhavnagar, is less explored_ in the bacterial diversity study and particularly for the EPS production from such organisms, thus the purpose was to analyze the physico-chemical properties of the collected samples, elucidation of the culturable diversity of marine bacteria isolated from the Alang coastline and their potential to produce EPS.

MATERIALS AND METHODS

Sampling site and sample collection

Ten different samples comprising five sea water (sample 1-5) and five sediments (sample 6-10) were collected for the study from the different sites (about 50m distance between two points) of the coastal region of Alang, Bhavnagar, having area of 160 x10 m (latitude 21°36' N, longitude 72°18'E). The surface water samples were collected directly into sterile bottles while the sediments (0.5-1.0m depths from water surface) were collected in sterile plastic bags.

Physico-chemical analysis of the sediment and water samples

From the collected water samples, pH and conductivity were measured directly using a portable multimeter analyzer (Eutech, Singapore). Where as for the collected sediment samples, 10% w/v sediments were suspended in distilled water for all the analysis. Parameters like total solids (TS) and total dissolved solids (TDS) were determined using gravimetry method. Soluble chloride estimation was done by titration with $AgNO_3$, Ca^{2+} and Mg^{2+} were measured using EDTA. Standard analytical procedures were followed for the determination of all the parameters **(APHA, 1995)**.

Isolation and characterization of marine bacteria

Collected samples were serially diluted and spreaded on Zobell Marine Agar (ZMA), Zobell Marine Sea Water Agar (ZMSA), R2A Agar, Artificial Sea Water Agar (ASWA) with 0.5 g/L peptone and 1.0 g/L glucose, Nutrient Agar (NA) with five different concentrations of sodium chloride (3, 5, 10, 15 and 20% w/v) and Alang Sea Water Medium (ASWM) containing 0.5 g/L peptone and 1.0 g/L glucose in sea water. All the media constituents used in the study were acquired from Hi Media, India. All the plates were incubated at 30±2 °C for 48 -72h to grow bacteria. The viable bacterial count was determined in terms of Colony Forming Unit (CFU) and from apparently different colonies, organisms were purified and sub cultured in the respective medium **(Bianchi *et al*., 1992)**. Isolates were preliminary identified on the basis of morphological and biochemical observations according to the methods described in the Bergey's manual of systematic bacteriology **(Brenner *et al*., 2005; Vos *et al*., 2009)**.

Salt tolerance study

Salt tolerance study of all the bacterial isolates was carried out using nutrient broth with increasing NaCl concentrations (0-30% w/v NaCl). In each tube containing 5.0 mL of the growth medium, 100 µL of activated bacterial culture (OD_{620}=1.0) was inoculated. Growth was measured at 620 nm after 24 h, 48 h and 72 h. Based on the salt tolerance potential all the isolates were categorized as non-halophiles, halotolerant, slight and moderate halophiles as described in The Prokaryotes **(Oren, 2006)**.

Diversity indices and statistical analysis

Based on the phenotypic characteristics of the organisms, Shannon Weiner diversity index (H'), Richness ($R_{margalef}$, $R_{menhinik}$) and Evenness (E_{Pielou}) were calculated by the standard formula **(Derry *et al*., 1998; Dave *et al*., 2002)**. The data of biochemical tests were applied to Principal Component Analysis (PCA) and Hierarchial Cluster Analysis (HCA) using the statistical package (IBM SPSS Statistics, version 20, 2011) for windows **(Soni *et al*., 2002)**.

Molecular identification by 16S rRNA gene sequencing and phylogenetic analysis

The taxonomic identity of the selected bacterial isolates was confirmed by 16S rRNA gene sequencing along with the biochemical tests. The 16S rRNA sequences of the bacteria were submitted to the GenBank and accession numbers were obtained. All sequences were aligned with a multiple sequence alignment and phylogenetic tree of the isolates was constructed by the neighbor joining analysis using Kimura's two-parameter model using MEGA 6.0 **(Kimura, 1980; Saitou and Nei, 1987)**.

Screening of EPS producers

Bacterial isolates that produced high mucoid colonies were selected for further screening and their EPS production ability was checked using EPS broth medium **(Atlas, 1993)**.

EPS production and extraction

The growth of various isolates was harvested in sterilized normal saline, cell count was performed spectrophotometrically at 600 nm and 10% (v/v) inoculum consisting $1x10^8$ cells/mL was inoculated into 250 mL capacity Erlenmeyer flask containing 100 mL EPS broth medium consisting of (g/L): casein hydrolysate, 15; sodium acetate, 12, K_2HPO_4, 10; yeast extract, 5; L-–Cystine, 0.5; NaOH, 30 and sucrose, 50. The flasks were incubated on orbital shaker (Newtronics, India) rotating at 150 rpm at 30±2 °C. At different time intervals the viscosity of the medium was studied. Extraction of EPS was carried out using chilled acetone in 1:3 ratio **(Watanabe *et al*., 1999)**, and was kept at 4 °C for overnight. Precipitated EPS was separated by centrifugation at 10,000 g and wet weight of EPS was recorded **(Ashok *et al*., 2011; Razack *et al*., 2013)**. The separated EPS was dried at 65 °C in oven to get the constant dry weight.

RESULTS AND DISCUSSION

Physico-chemical characterization of samples

The physico-chemical characteristics of the samples are represented in Table 1. The pH of the samples ranged from 7.25 to 8.92 obviously due to the presence of dissolved salts in marine water. The temperature at the time of sample collection was 34±4 °C. The conductivity of the samples ranged from 9.4 mS to 76.8 mS. The sediment samples showed salinity ranging from 3570-18400 mg/L, whereas, water samples showed the salinity ranging from 21950-35100 mg/L. The chloride (Cl^-) concentration ranged from 2170-10250 mg/L and 12053-22688 mg/L for sediment samples and water samples respectively. NaCl concentration of sediment samples ranged from 2095.5-16912.5 mg/L and that of water samples ranged from 866.25-2179.55 mg/L. Hardness of the samples in terms of Ca^{2+} and Mg^{2+} was found in the range from 78-758 mg/L and 320-1200 mg/L respectively. Samples had TDS values between 15120-53100 mg/L. The overall composition of the samples differed depending on the site of sampling. Physico-chemical analysis of the liquid samples showed minor pH variations as compared to solid samples. However, conductivity, TS, salinity, Cl^-, Mg^{2+}, Ca^{2+} content of water sample showed variation of 1.73, 1.72, 1.80, 1.88, 1.51, 1.66 fold respectively. Where as conductivity, TS, salinity, Cl^-, Mg^{2+}, Ca^{2+} content of sediment sample showed variation of 3.98, 2.41, 4.74, 4.72, 5.48, 1.62 fold respectively, which were analyzed by preparing the 10% samples. However, the results presented here are of one particular day and hence the variation was due to the different sites that too within 160x10m are and not due to the season. Sediment systems were more diverse as compared to water samples collected from the same place. As per the reported results of the coastal region of Alang and South Saurashtra coastal stretch of Gujarat, pH ranges from 7.9 to 8.39 **(Bhadeja and Kundu, 2011)**.

Table 1 Physico-chemical analysis of the samples

Samples	pH	Conductivity (mS)	TS (mg/L)	TDS (mg/L)	Salinity (mg/L)	Cl^- (mg/L)	Ca^{2+} (mg/L)	Mg^{2+} (mg/L)	NaCl (mg/L)
1	7.35	58.92	44450	44350	30240	21979	608	880	36265.35
2	7.25	69.55	48814	48750	35100	22688	588	1050	37435.2
3	7.45	76.8	53232	53100	19775	12053	758	1200	19887.45
4	7.56	48.39	37592	37520	27360	14889	488	850	24566.85
5	7.25	44.15	30896	30800	21950	15598	456	790	25736.7
6	8.62	12.06	30400	29800	3570	2170	318	480	2095.5
7	8.92	9.4	26350	25900	4090	3210	119	369	5296.5
8	8.75	11.8	37250	36500	6390	4180	428	520	6732
9	8.33	37.5	29050	28500	18400	10250	268	460	16912.5
10	8.01	14.19	15420	15120	8370	5250	78	320	8662.5

1-5 water and 6-10 sediment samples

Morphological and physiological characterization of isolates

Initially sample number 4, 6 and 8 were studied on all the 10 culture media described above to select the most suitable medium for the study of all the samples. Detail of the bacterial viable count of these samples is given in Table 2a. Amongst all the media used, the highest bacterial count was obtained on ZMA, and Nutrient agar with 3% NaCl concentrations, respectively, hence further isolation study from all the samples was carried out with ZMA and NA (both with 3% NaCl) and the CFU of bacteria on ZMA in comparison with NA is listed in Table 2b. The highest CFU/mL of culturable bacteria was found as $134x10^6$ in the sample number 8 on ZMA; whereas, the lowest CFU/mL of culturable bacteria was found as $0.5x10^6$ in the sample number 5 on NA . ZMA proved to be the medium of choice for the isolation of the organisms from such saline habitat. As can be seen from the results some of the bacteria grew even in the presence of 20% NaCl concentration in NA(Table 2a), indicated the presence of helophilic or helotolerant organisms.

Table 2a Total bacterial count of three samples on all media studied

Sample	Total viable count ($x10^6$ CFU/ml)									
	Medium									
	ZMA	ZMSA	R2A	ASWA	ASWM	NA (3%)	NA (5%)	NA (10%)	NA (15%)	NA (20%)
4	2.2	0.68	0.021	0.024	0.028	0.8	0.74	2*	0.012*	0.01*
6	84	62	0.044	0.05	0.044	24	0.88	4.7*	0.03*	0.024*
8	134	40	0.052	0.062	0.088	32	1.2	7.0*	0.021*	0.026*

*= _$x10^3$ CFU/mL

Table 2b The viable count of bacteria on ZMA in comparison with NA.

Medium	Total viable count (x10^6 CFU/mL)									
	Sample number									
	1	2	3	4	5	6	7	8	9	10
ZMA	6.8	3.1	4.4	2.2	1.2	84	40	134	12	7
NA	3.2	2.5	1.1	0.8	0.5	24	13	32	40	20

Morphologically different 141 bacterial isolates were isolated from the collected samples. The colony size of the isolates ranged from 0.4 to 2.8 mm. Out of 141 isolates, 105 were gram positive and 32 isolates were gram negative and 4 were actinomycetes. In case of gram positive isolates, cocci were large in numbers as compared to gram positive rods. Out of 105 gram positive isolates, 55% were cocci, 41% were gram positive rods. Amongst 141 isolates, 23% of the isolates were able to produce varieties of pigments while the rest of them gave nonpigmented colonies. Pigmented colonies showed white, creamish white, yellow, red, orange, pink and bluish green pigments. Yellow pigmented colonies showed variation in shades like golden yellow, lemon yellow and light yellow. Comparison of pigmented and non-pigmented colony from all the samples studied is shown in Fig.1a. Sample 8 showed the highest percent of pigmented isolates and sample 10 showed the lowest percent of pigmented isolates. Light yellow pigments were the highest in number where as bluish green was the lowest. Gram positive cocci produced variety of pigments as compared to gram positive bacilli and gram negative rods (Fig. 1b).

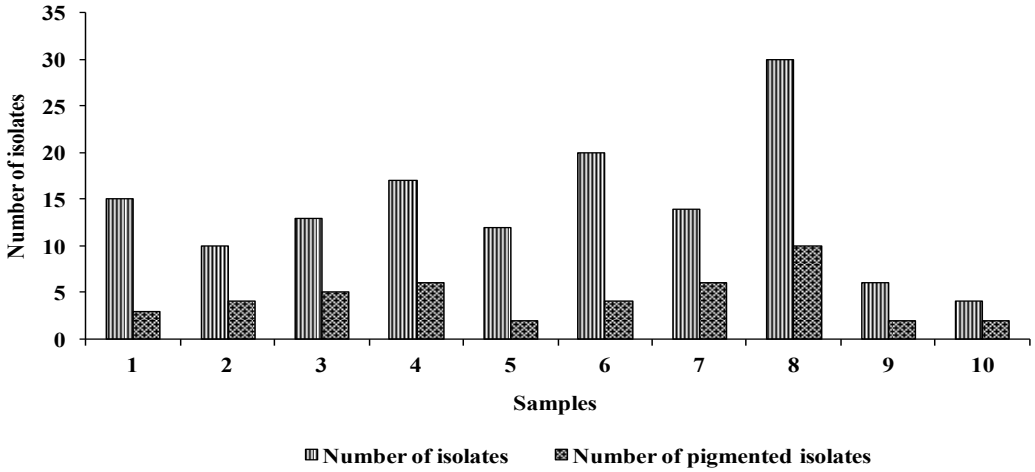

Figure 1a Comparison of pigmented bacteria with the total number of isolates in different samples

Amongst 141 bacterial isolates, 132 isolates were able to grow up to 5% of NaCl concentration, 105 isolates up to 10%, 27 isolates up to 15% and only 5 isolates showed growth up to 20% of salt concentration. From these five, only two were able to grow up to 25% of NaCl concentration. None of them showed any growth at 30% w/v of salt concentration. As the salt concentration was increased the growth of the organisms was found to be decreased into the medium. Organisms showed intense growth at low salt concentrations (3-5%) within 24-48 h, moderate growth at 10% of NaCl concentration and scanty growth at high NaCl concentration (15-25%) after 2-3 d of incubation period. There are some reports on the diversity study of marine salterns near Bhavnagar and from the coastal region of Dwarka-Veraval. According to that the microbial diversity and growth of the microorganisms found to decrease with higher salt concentration **(Dave and Desai, 2006; Nanjani and Soni, 2012)**. Gram positive bacteria were more in numbers between 10-15% of NaCl concentrations; whereas, gram negative isolates showed dominance between 20-25% of NaCl concentrations. The comparable results are also obtained in a study of isolation of halotolerant and halophilic bacteria **(Purohit *et al.*, 2015)**.

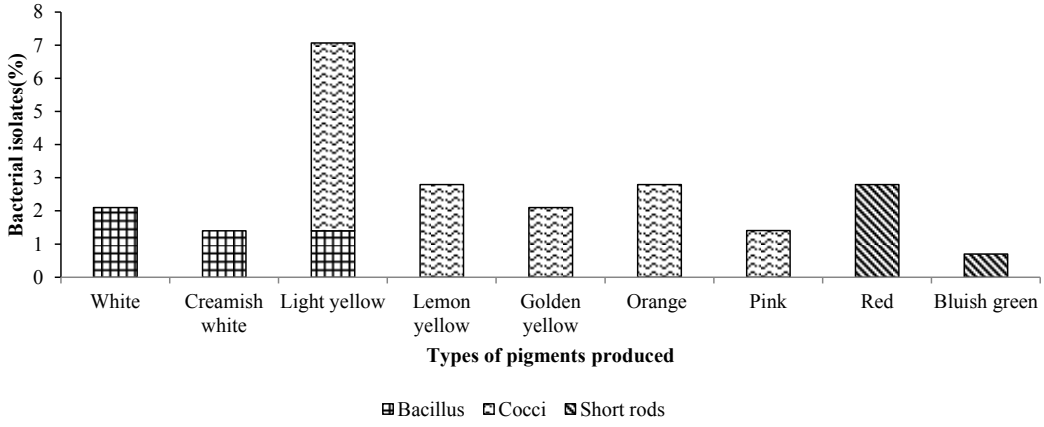

Figure 1b Dominance of the group of bacteria, in particular pigment production

Diversity indices

The results of various diversity indices are presented in Table 3. As can be seen for all the samples, Shannon Weiner index (H') varied from 1.043 to 2.86. Sample 3, 6 and 8 had H'values above 2.5. Sample 1, 2 and 4 had H' values above 2, forming another group. The rest of the samples showing H' index values above 1 were grouped separately. Evenness values ranged from 0.752 to 1.209. Richness, $R_{margalef}$ ranged from 0.333 to 1.140 and $R_{menhinik}$ ranged from 0.0422 to 0.0848. Based on all the four indices, it can be concluded that the sample number 10 had overall low diversity where as sample 8 showed high diversity, high richness and high evenness. Moreover, it represented the most diverse bacterial communities capable of growing with a wide range of salt concentrations and producing variety of pigments could be the reason for its high diversity value. The lowest dissolved solids, Ca^{2+} and Mg^{2+} content could be responsible for low diversity and evenness of the organisms in sample 10. However, there are some samples with high diversity and richness, but low evenness and vise versa. **Haque *et al.*, (2004)** have reported that the samples, which shows similar diversity values may incorporate low evenness and the high richness or consist of high evenness and low richness. In some cases the sample shows all three parameters high or low simultaneously. Therefore, one should consider the diversity, richness and evenness collectively during diversity studies.

Table 3 Diversity indices for bacterial populations from various samples based on their physiological profile

Sample no.	Shannon Diversity (H')	Richness		Evenness
		$R_{Margalef}$	$R_{Menhinick}$	E_{Pielou}
1	2.47	1.11	0.0848	0.994
2	2.15	0.723	0.0632	1.034
3	2.51	0.696	0.0527	1.209
4	2.09	0.844	0.0789	0.952
5	1.54	0.418	0.0422	0.96
6	2.73	0.87	0.0567	1.189
7	1.41	0.549	0.0632	0.787
8	2.86	1.140	0.0675	1.11
9	1.55	0.537	0.0572	0.866
10	1.043	0.333	0.0447	0.752

Principal component and Hierarchical Cluster Analysis

PCA based on different biochemical tests was able to group the 39 bacterial isolates on the basis of the similarities and differences in their metabolic behaviour. Results are shown in the Fig. 2a and 2b. Total 7 principal components were extracted. PC1 and PC2 explained 18.84% and 14.19% diversity, respectively, of the total variance present in the original data. Bacterial isolates formed different groups in PCA, among these groups isolates E2 and E3, SR31 and SR 103, SR 49, SR52 and KD, SR 24, K5 and K8, SR 40 and SR 47 were closely placed because of their similar metabolic behaviour. The Hierarchical Cluster Analysis represents the similar observations as PCA. It shows the least distance between the isolates explained above. Isolate SR 104 placed distinctly from the rest of the isolates in PCA. It could be due to its distinct metabolic properties . Isolates also showed a wide diversity With respect to fermentation of 21 sugars studied(Results are not shown).

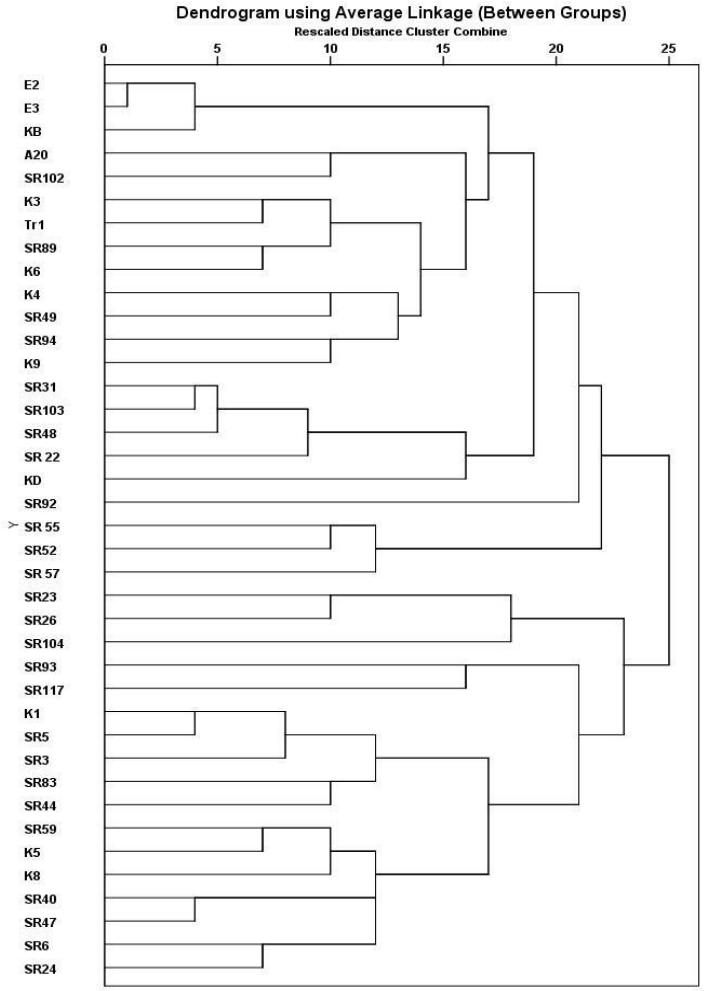

Figure 2a The Dendogram showing relationship amongst bacterial isolates using Hierarchical cluster analysis of biochemical test data

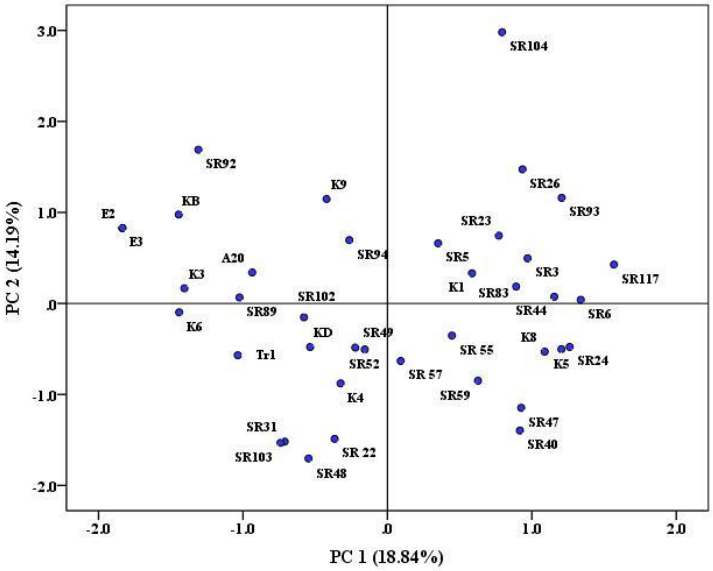

Figure 2b PCA of biochemical tests of 39 isolates at their optimal incubation time

Phylogenetic analysis of the isolates

Based on the results of 16S rRNA partial gene analysis selected isolates represented 22 genera and various species of these genera. Thus, it is obvious to have morphological, metabolical and EPS production diversity. Phylogenetic analysis of the isolates showed that most of them belonged to the phylum Firmicutes, followed by gamma-Proteobacteria. Gram positive bacteria of genera *Bacillus, Exiguobacterium, Micrococcus, Arthrobacter, Kocurea, Planococcus, Cellulosimicrobium, Actinotalea* and some of the Gram negative isolates related to genera *Pseudomonas, Enterobacter, Pontibacter, Sinorhizobium, Mesorhizobium* and *Dyadobacter* were cultured from the Alang ecosystem. Gamma-Proteobacteria included isolates of *Halomonas* and *Marinobacter* sp. The studied samples were from the same plot at the Alang coastal line, but they are from 10 different sites, which could be the reason of great diversity observed in the bacterial isolates. Members of the genus *Bacillus, Exiguobacterium, Micrococcus, Arthrobacter, Planococcus, Cellulosimicrobium, Pseudomonas* and *Enterobacter* have also been isolated from other saline environments, including Krishna Godavari basin (Bay of Bengal), Lonar Lake (Maharashtra) and coastal area of Tamilnadu **(Jaynath *et al.*, 2002; Kanekar *et al.*, 2007; Devi *et al.*, 2011)**. Based on the 16S rRNA gene analysis, evolutionary relationship between the cultivable microbes, isolated from the coastal region of Alang, Bhavnagar was studied using the neighbor- joining method (Fig. 3). The accession numbers are given in parentheses. Only bootstrap values are shown at nodes (based on 500 bootstrap resampling). The scale bar represents 1% divergence. Total 41 different microbial species were identified. The evolutionary distance is depicted by the length of the horizontal line. The branch point along the length of the horizontal line is the point of divergence of two microbial species. The outer, *Paenibacillus polymyxa* represents a distinct group with minimum evolution in relationship with the rest of the isolates. On the other hand, the significant divergence (2 to 3 step divergence) is observed among other microbes, including halotolerant *Halomonas*, chemoorganotrophs *Salinicola salaries*, aerobes *Pseudomonas*, anaerobes and facultative anaerobes *Enterobacter*. The community also contains some unique plant pathogens like *Xanthomonas campestris*. On the basis of this diversity study, it can be concluded that the selected site is highly enriched with the various types of microbes having divergent metabolic potential in spite of ship breaking activity at the site.

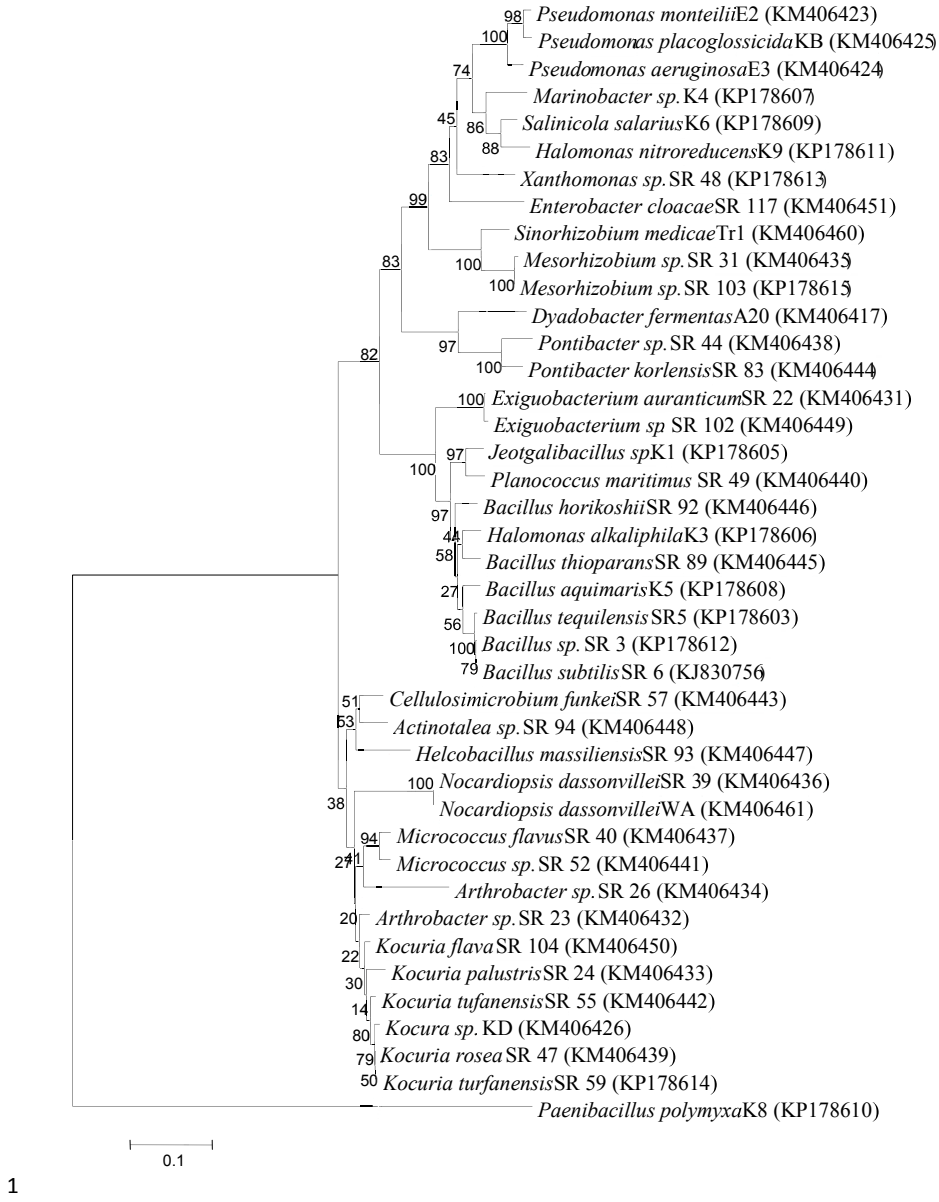

Figure 3 Neighbor joining phylogenetic tree of the 16S rRNA partial gene sequences of bacteria isolated from the coastal region of Alang, Bhavnagar

Rheological properties and EPS production study

Change in viscosity of the media was measured, in which EPS producers were grown and studied organisms showed quite a broad variation in viscosity. Out of 141 isolates 88 isolates showed variation in viscosity between $9.20x10^{-6}$ to $1.6x10^{-5}$ m.pa.s^{-1}, 28 isolates showed it between $1.7x10^{-5}$ to $2.6x10^{-5}$ m.pa.s^{-1} and for remaining 25 isolates it was $2.7x10^{-5}$ to $2.09x10^{-4}$ m.pa.s^{-1} when cultivated in EPS broth medium. Isolate SR5, SR6, SR40, SR48, SR55, SR83, SR117, Tr1 and K9 were found to be high EPS producers. In this study EPS production was observed in the range from 0.76 g/L to 10.7 g/L. The observed variation was obviously due to the diverse variety of the species as well as genus. EPS production yield by *Halomonas nitroreducens* was 0.76 g/L, *Sinorhizobium medicae* was 3.44 g/L, *Enterobacter cloacae* was 4.2 g/L, *Xanthonas* sp. was 6.48 g/L, *Kocurea turfenensis* was 7.1 g/L, *Micrococcus flavus* was 8.0 g/L, *Bacillus tequilensis* was 8.8 g/L, *Pontibacter korlensis* was 9.8 g/L, and *Bacillus subtilis* was 10.7 g/L. Reported range of EPS production from halotolerant organism is 0.16 g/L to 3.0 g/L. *Halomonas ventosa*, *Bacillus licheniformis, Halomonas anticariensis* are moderate halophiles showing EPS yield 0.29 g/L, 0.165 g/L and 0.5 g/L respectively **(Bejar *et al*., 2006; Maugeri *et al*., 2002)**. As halotolerant organisms survive in extreme conditions, they may be used for production of biopolymers, halophilic enzymes, compatible solutes and in the bioremediation process **(Ventosa *et al*., 1998; Margesin and Schinner, 2001; Mellado and Ventosa, 2003)**.

CONCLUSIONS

The site of Alang could be a good source of culturable bacterial diversity as different halotolerant and moderate halophilic bacteria were isolated from it. To the best of the authors' knowledge, the presence of the organisms, namely *Pontibacter korlensis, Kocurea tirfenensis, Actinotalea, Sinorhizobium* sp., *Cellulosimicrobium funkei, Helcobacillus massiliensis* and *Dyadobacter fermentas* from the coastal area of Alang, Bhavnagar is the first report. The significant finding was that many of these isolates grew in the presence of as high as 3-10% NaCl concentrations and some grew even in 20-25% NaCl, more over they produce a significant amount of EPS. EPS production by *Kocurea turfenensis* (SR55) and *Pontibacter korlensis* (SR 83) might be the new finding of this study. EPS produced by these organisms can be explored for various economical uses as it may have distinct properties with biotechnological applications.

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IMPLICATIONS OF ANTIBIOTIC RESISTANCES PRODUCED BY PHENOTHIAZINES IN *Mycobacterium tuberculosis*

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ABSTRACT

Several chemotherapeutic agents applied to human beings for past few decades for different ailments, have been found to possess potent antitubercular activity. Two such agents, methdilazine (Md) and promethazine (Pz) were used to select mycobacterial mutants resistant to themselves at different levels and tested to determine if such mutants simultaneously developed cross-resistance to known antitubercular drugs. Mutants were produced by application of a heavy inoculum on Lowenstein-Jensen medium containing Md (or Pz) at concentrations higher than their respective minimum inhibitory concentrations (MICs). These were then tested to find out if such mutants have changed their resistogramme pattern with respect to the test antitubercular agents. Certain first-step Md-mutants became simultaneously resistant to ethambutol and pyrazinamide; while the step-2 Md-mutants revealed further increase in resistance to these agents along with resistance to isoniazid, rifampicin and streptomycin as well. In the study with Pz it was noted that many mutants showed distinctly higher levels of resistance to all the test drugs, particularly to isoniazid, pyrazinamide and streptomycin. The levels of MIC were distinctly high in many mutants. These observations on cross-resistances seem to be best explained on the basis of a reduction in cell-membrane permeability acting in a non-specific manner. The role of such a cross-resistance may possibly be accounting for an overall increase in the MICs of many drugs against several groups of microorganisms including *Mycobacterium tuberculosis* and other species of *Mycobacterium* during the last five decades.

Keywords: Cross-resistance; *Mycobacterium tuberculosis*; phenothiazines; mutants; antitubercular agents

INTRODUCTION

Tuberculosis is a remarkably high risk communicable disease of human beings. The causative organism *Mycobacterium tuberculosis* is airborne and is often transmitted among people of lower income group suffering from malnutrition and immunological deficiencies. The World Health Organization (**WHO, 2013**) reported a total of 8.6 million new cases of tuberculosis and 1.3 million deaths due to this infection in 2012. In the treatment regime referred as Directly Observed Treatment Schedule (DOTS) isoniazid, rifampicin, ethambutol and pyrazinamide are administered for two months after presumptive diagnosis. Subsequently isoniazid and rifampicin are continued in the same patients for 4 to 7 months depending on the severity of infection. However, now there are more virulent forms designated as multi-drug resistant strains that are resistant to isoniazid and rifampicin. Apart from this many strains are found to be resistant to isoniazid, rifampicin, streptomycin, any fluoroquinilone plus any of the antitubercular injectables like amikacin/ kanamycin/ capreomycin. Such strains have evolved due to misuse or overuse of the scheduled drugs or failure in continuation of the correct therapy. These strains may also arise if the treatment schedule is allowed to continue for more than 12 months. Simultaneous application of so many drugs creates sufficient pressure for the causative organism to select multi-drug resistant mutants. Such a situation could not have occurred by a simple mode of action since different drugs have different sites of action. The modes of action of anti-tubercular drugs are very varied, although structurally similar drugs usually have the same mutated target.

The occurrence and prevalence of tuberculosis by drug resistant organisms initiated systematic search for antimycobacterial agents from various existing pharmacological agents by several groups of researchers in different parts of the world. Such studies revealed that antipsychotic and antihistaminic phenothiazines possess powerful antitubercular action. Most potent among these were thioridazine, methdilazine, trifluoperazine, chlorpromazine and promethazine (**Molnar et al., 1977; Kristiansen and Vergemann, 1986; Ratnakar and Murthy, 1993; Chakrabarty et al., 1993; Dutta et al., 2009; Crowle et al., 1992; Amaral et al.,1996; van Ingen et al., 2009; Advani et al., 2012; Kristiansen et al., 2015**). These were reported to be simultaneously active against a large number of Gram positive and Gram negative bacteria as well (**Kristiansen, 1979; Radhakrishnan et al., 1999; Mazumdar et al., 2001; Dastidar et al., 1995; Dastidar et al., 2004; Dastidar et al., 2013**).

Occurrence of cross-resistances among aminoglycosides in *Mycobacterium tuberculosis* was reported as far back as 1959 by several workers (**Torii et al., 1959; Tsukamura, 1959**). **Koseki & Okamoto (1963)** presented evidences for a significant change in resistance to viomycin caused by development of resistance to capreomycin in *Mycobacterium tuberculosis*. **Tsukamura (1969)** while trying to produce drug resistant mutants in the laboratory found that highly kanamycin resistant *M.tuberculosis* strains were resistant to capreomycin and strains moderately resistant to kanamycin were susceptible to capreomycin. He further observed that experimentally produced capreomycin resistant strains failed to develop resistance to kanamycin. Such a one way cross-resistance relationship was observed among many other aminoglycosides **(Tsukamura,1974)**. **Tsukamura and Mizuno (1975)** while trying to determine cross-resistance relationships among aminoglycosides in *M.tuberculosis* reported that resistances to several antibiotics could be produced by a single mutation to any one of the agents. In **2005 Maus et al** attempted to analyze cross-resistance to capreomycin, kanamycin, amikacin and viomycin in *M.tuberculosis* at molecular level. According to these authors mutation of the *thyA* gene confer capreomycin and viomycin resistance in *M.tuberculosis*. It is known that in mutations in the 16S rRNA gene (*rrs*) have been associated with resistance to all these four drugs (**Suzuki et al., 1998**). **Maus et al (2005)** reported three *rrs* mutations in their *M.tuberculosis* test strains each of which was associated with a particular cross-resistance pattern. They opined that when *M.tuberculosis* strains are exposed to one or two drugs phenotypic and genotypic differences can be seen in the development of antibiotic cross-resistance.

In the present study we have tried to determine the ability of antitubercular phenothiazine compounds methdilazine and promethazine to produce mutants resistant to themselves and also to detect if such mutants develop cross-resistances to known antitubercular agents.

MATERIAL AND METHODS

Strains

M.smegmatis 789, *M.phlei* L1, *M. avium 724, M.flavescens 1541, M. gordonae 1324, M.intracellulare* 1406, *M.tuberculosis* H_{37}Rv 102 and *M.tuberculosis* H_{37}Ra 16 were obtained from Dr V.M. Katoch, the then Director of National JALMA Institute for Leprosy and other Mycobacterial Diseases, Agra, India. Remaining three strains *M.tuberculosis* Bajaj 1, J15, and N23 were obtained from Dr A.N. Chakrabarty, Department of Medical Microbiology & Parasitology, Calcutta University College of Medicine, Kolkata, India. All the organisms were received as live culture slants in Lowenstein-Jensen Medium (LJM) from both the institutions and maintained in the same medium throughout the study.

Media

All the biological components were obtained from Oxoid (UK). Kirchner's Liquid Medium (KLM) and LJM were prepared as per established protocol (**Barrow and Feltham, 2003**).The growth was confirmed for *Mycobacterium* spp. after performing Z-N staining and different biochemical tests, like niacin test, nitrate reduction test and catalase test (**Kamerbeek et al., 1997**).

Overlay medium

This was prepared with 7H10 agar base, distilled water and glycerol, distributed in 2mL amounts and sterilized by autoclaving.

Inoculation

The known standard strains *M.tuberculosis* H_{37}Rv 102 and H_{37}Ra 16 along with all the other mycobacteria were grown in KLM, vortexed, diluted and standardized. Mcfarland standard 0.5 (in turbidity standard; the turbidity standard was prepared by adding 0.5 ml of a barium chloride solution to 99.5 ml of 1% H_2SO_4) was routinely taken for inoculation of all the strains and their mutants.

Media containing antitubercular drugs and other selecting agents

The agents were obtained in pure dry powder form from their manufacturers in India and stored at 4°C. To 2ml of overlay medium was added, any of the following agents: isoniazid acid hydrazide (INH), rifampicin (Rf), ethambutol (Eb), pyrazinamide (Pz), streptomycin (Sm), methdilazine (Md) and promethazine (Pz). The final concentration (µg/ml) of each of the agents were: 1,2,5,10,25,50,100, 200,400, 800, 1000 and 2000; these were added to the overlay medium before being allowed to flow over the freshly prepared LJM slants. All such bottles were inoculated for appearance of growth.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC of the agents for different strains of *Mycobacterium* and their mutants were determined by inoculating the media as described above and incubating at 37°C. Appearance of growth was checked every day up to 3 weeks. Adequate strain and media controls were run throughout the tests.

Production and isolation of Md and Pz mutants

This was done by application of a heavy inoculum on LJM containing Md, the amount being higher than the MIC of the organism with respect to the agent. As the mutants developed they were designed as Step 1 mutants and were inoculated on LJM containing still higher amount of Md. The colonies developing on such a medium were designated as Step 2 mutant. In this way higher steps of mutants were selected with both Md and Pz. The relationship of the mutants with respect to the original wild types was determined with the help of various known and standardized morphological and biochemical parameters as described earlier.

RESULTS

Sensitivity of bacteria

The minimum inhibitory concentration (MIC) of INH, Rf, Eb, Py,Sm,Md nd Pz with respect to 20 strains of *Mycobacterium* is presented on Table 1. These strains were selected on the basis of previous studies that had revealed that most of these were sensitive to the test drugs including the phenothiazines at low concentrations. The MIC of INH, Py and Sm in the recent clinical isolates *M.tuberculosis* Bajaj 1, J15 and N23 was rather high. In *M.tuberculosis* Bajaj 1, the MIC of INH and Sm was 25µg/ml. The strain N23 was even more resistant, the MIC of Py and Sm was as high as 50µg/ml. With respect to the phenothiazines, the MIC values ranged between 10 and 25µg/ml in most of the test strains; however, in case of Bajaj 1, J15 and N23 the MIC of Md and Pz was 50µg/ml.

Table 1 Minimum inhibitory concentration (MIC) of anti-tubercular drugs, methdilazine and promethazine against various strains of *Mycobacterium*

Mycobacterium	MIC (µg/ml)						
	INH	Rf	Eb	Py	Sm	Md	Pz
M.smegmatis 789	2	2	2	5	2	25	10
M.smegmatis 1546	2	2	2	5	2	10	25
M.fortuitum 1529	2	2	2	2	5	10	25
M.scrofulaceum 1323	2	1	2	2	2	10	10
M.avium 724	5	5	10	2	10	25	25
M.gordonae 1324	2	2	2	5	2	10	10
M.phlei L1	5	2	2	10	2	25	25
M.marinum 50	2	2	5	5	2	25	25
M.intracellulare 1406	5	5	5	5	2	10	25
M.flavescens 1541	2	2	5	5	2	10	10
M.terrae 1450	5	2	2	5	2	10	10
M.tuberculosis H_{37}Ra16	2	2	2	5	2	5	25
M.tuberculosis H_{37}Rv102	2	2	5	5	2	10	25
M.tuberculosis Bajaj1	25	5	5	5	25	50	50
M.tuberculosis J15	25	5	10	25	25	50	50
M.tuberculosis N23	25	5	5	50	50	50	50
M.tuberculosis K1	2	1	2	5	2	10	25
M.tuberculosis 2	2	1	5	2	2	10	25
ICRC bacillus	2	2	2	5	2	10	10
"Skinsness" bacillus	2	2	5	5	2	10	25

INH, isoniazid acid hydrazide; Rf, rifampicin; Eb, ethambutol; Py, pyrazinamide; Sm, streptomycin; Md, methdilazine; Pz, promethazine

Selection of mutants

All the 20 strains of *Mycobacterium* spp. were investigated for production of highly resistant Md mutants; of these 11 failed to produce mutants even after repeated tests. Nine strains of mycobacteria passaged on low but step-wise gradually rising concentrations of Md finally produced mutants of high resistance. The mutants were accepted only when the identification tests particularly with respect to cultural morphology in LJM coupled with acid fastness, established that they belonged to the respective wild-types. Following the same principle Pz mutants were selected. It may be pointed out here that *M. tuberculosis* Bajaj 1, J15, N23, H_{37}Rv102 and H_{37}Ra16 were able to develop fairly resistant mutants with respect to both Md and Pz. Among the others *M.phlei* L1, *M.flavescens* 1541, *M.avium* 724 and *M.gordonae* 1324 were able to produce Md- resistant mutants while 4 other strains of *Mycobacterium* could successfully develop Pz resistant mutants. The remaining 7 strains were unable to select drug resistant mutants.

Antibiotic cross-resistance patterns of Md and Pz-resistant mutants

It may be noted from Table 2 that in *M phlei* L1 the MIC of Md was 25 µg/ml, the first step mutant could be developed at 50 µg/ml of Md when the MIC of Md was found to be 100 µg/ml. From this the step 2 mutant of the same organism was developed whose MIC value of Md was 400 µg/ml. There was a gradual increase in the MIC values with respect to INH, Rf, Eb and Py while there was no change in MIC values with respect to Sm. A similar pattern was noted in *M.flavescens* 1541. However, in the Md- mutants of *M.avium* 724 and *M.gordonae* 1324, MIC values decreased in case of Sm and Py respectively. *M. tuberculosis* $H_{37}Rv$ 102 and Ra 16 being rather sensitive to test drugs, the levels of Md resistances in mutants of these strains were not so high and the changes in resistogramme pattern of mutants were not significant (Table 2). On the contrary, *M. tuberculosis* Bajaj 1, J15 and N23 being much less sensitive to the test agents, produced mutants at much higher levels of Md and simultaneously exhibited greater MICs with respect to all the antitubercular drugs except the strain N23 whose MIC value of Eb decreased in the mutants.

Table 2 Changing pattern of resistances of antimycobacterial drugs in methdilazine resistant mutants of *Mycobacterium* spp.

Mycobacteria	Types of cultures	MIC (µg/ml in LJM) of different agents for the wild type and mutant bacteria					
		INH	Rf	Eb	Py	Sm	Md
M.phlei L1	Wild Type	5	2	2	10	2	25
	Md mutant step 1 (50)	5	2	2	10	2	100
	Md mutant step 2 (200)	10	10	5	25	2	400
M.flavescens 1541	Wild Type	2	2	5	5	2	10
	Md mutant step 1 (25)	5	2	5	25	10	100
	Md mutant step 2(200)	5	2	10	25	25	500
M.avium 724	Wild Type	5	5	10	2	10	25
	Md mutant step 1 (50)	5	10	25	5	10	200
	Md mutant step 2(400)	5	10	25	5	5	100
M.gordonae 1324	Wild Type	2	2	2	5	2	10
	Md mutant step 1 (50)	2	2	2	2	2	200
	Md mutant step 2(400)	5	2	5	2	5	1000
M.tuberculosis Bajaj1	Wild Type	25	5	5	5	25	50
	Md mutant step 1 (100)	25	5	10	10	25	200
	Md mutant step 2(400)	50	5	10	25	25	500
M.tuberculosis J15	Wild Type	25	5	10	25	25	50
	Md mutant step 1 (200)	25	5	25	10	25	500
	Md mutant step 2(1000)	50	5	25	10	25	2000
M.tuberculosis N23	Wild Type	25	5	5	50	50	50
	Md mutant step 1 (200)	50	5	2	50	50	500
	Md mutant step 2(1000)	100	5	2	100	50	2000
M.tuberculosis $H_{37}Rv102$	Wild Type	2	2	5	5	2	10
	Md mutant step 1 (25)	2	2	10	5	5	100
	Md mutant step 2(200)	2	2	10	25	5	500
M.tuberculosis $H_{37}Ra16$	Wild Type	2	2	2	5	2	5
	Md mutant step 1 (25)	2	5	2	5	5	50
	Md mutant step 2(100)	5	5	2	2	5	200

Table 3 Promethazine (Pz) resistant mutants of *Mycobacterium* spp. and their effects on change of resistant patterns with respect to anti-mycobacterial drugs

Mycobacteria	Types of cultures	MIC (µg/ml in LJM) of different agents with respect to wild type and mutant bacteria					
		INH	Rf	Eb	Py	Sm	Pz
M.fortuitum 1529	Wild Type	2	2	2	2	5	25
	Pz mutant step 1 (50)	5	2	2	5	5	100
	Pz mutant step 2 (200)	5	2	5	25	10	400
M.scrofulaceum 1323	Wild Type	2	1	2	2	2	10
	Pz mutant step 1 (25)	5	1	2	25	10	50
	Pz mutant step 2(100)	5	2	10	25	25	400
M.marinum 50	Wild Type	2	2	5	5	2	25
	Pz mutant step 1 (50)	5	5	25	5	5	100
	Pz mutant step 2(200)	5	10	25	5	5	400
	Pz mutant step 3(500)	10	25	50	10	5	1000
M.intracellulare 1406	Wild Type	5	5	5	5	2	25
	Pz mutant step 1 (50)	5	2	5	5	2	200
	Pz mutant step 2(400)	5	2	10	10	5	500
	Pz mutant step 3(1000)	25	2	50	25	10	2000
M.tuberculosis Bajaj1	Wild Type	25	5	5	5	25	50
	Pz mutant step 1 (100)	50	5	5	10	25	200
	Pz mutant step 2(400)	100	5	10	25	50	1000
M.tuberculosis J15	Wild Type	25	5	10	25	25	50
	Pz mutant step 1 (100)	25	10	25	10	50	200
	Pz mutant step 2(400)	25	10	50	10	50	1000
M.tuberculosis N23	Wild Type	25	5	5	50	50	50
	Pz mutant step 1 (100)	25	5	5	50	100	400
	Pz mutant step 2(500)	100	5	10	100	100	1000
M.tuberculosis $H_{37}Rv102$	Wild Type	2	2	5	5	2	10
	Pz mutant step 1 (25)	5	2	5	10	2	50
	Pz mutant step 2(100)	5	2	10	25	10	400
M.tuberculosis $H_{37}Ra16$	Wild Type	2	2	2	5	2	5
	Pz mutant step 1 (10)	2	2	2	5	2	25
	Pz mutant step 2(50)	10	5	5	5	5	100

The step-wise mutants produced by mycobacteria against Pz showed nearly similar pattern of increase in resistances against the antitubercular drugs. However, in case of *M.intracellulare* 1406 there was a loss in the MIC value of Eb (Table 3).

DICUSSION

Prevalence of cross-resistances between aminoglycosides in drug-resistant mutants of *M.tuberculosis* has been studied extensively. Resistance to kanamycin in viomycin-resistant strains and resistance to streptomycin in kanamycin-resistant strains were two to four times greater than the resistant levels of the parent strains of *M.tuberculosis* (**Torii et al., 1959; Steenken et al., 1959**). **Tsukamura (1974)** isolated two types of tuberactinomycin N resistant mutants of *M.tuberculosis,* the first one was resistant to low levels of tuberactinomycin N, viomycin and capreomycin, while the other was resistant at high levels to all these three antibiotics plus kanamycin and lividomycin. With the help of an intensive study **Tsukamura and Mizuno** in **1975** proved that aminoglycoside antibiotics could be classified into three major types: streptomycin resistance, combined viomycin-tuberactinomycin N- capreomycin resistance, and the third type included resistance to kanamycin, lividomycin and paramomycin. No cross-resistance between streptomycin and any other amionoglycoside antibiotic was observed. Although there is a large number of studies on clinical isolates of *M.tuberculosis* on mutations of genes and their relatedness of resistances to specific antibiotics (**Taniguchi et al., 1996; Telenti et al., 1993; Ginsburg, 2005; Pitaksajjakul et al., 2005**) evaluation of drug-resistance in experimentally produced mutants of *M.tuberculosis* has not been reported during past several years.

Imperiale et al (2014) studied cross-resistances to isoniazid, rifampicin and levofloxacin at a molecular level in clinical isolates of *M.tuberculosis*. With the help of microplate colorimetric method they determined MIC of isoniazid, ethionamide, rifampicin, rifabutin and moxifloxacin in the clinical isolates. Mutations conferring drug resistances were detected by GenoType MTBDR plus and DNA sequences. Isoniazid and ethionamide cross resistance was detected in 95.12 % of isoniazid resistant isolates harbouring a mutation in inhAP or inhA open reading frame, but rifabutin cross-resistance was observed in 90% of clinical isolates originally shown to be resistant to rifampicin. This study highlighted that the same mutation causing resistance to the first line antitubercular drugs can be responsible for resistance to their respective structural analogs. Such findings are expected to help clinicians to decide on the treatment regime.

In this study it has been observed that Md mutants of *M.phlei* L1 developed cross-resistance to INH, Rf, Eb and Py in stepwise manner but not to Sm; with respect to *M.flavescens* 1541 the Md mutants developed cross-resistances to INH, Eb, Py, Sm and not To Rf. Wild type clinical isolates *M.tuberculosis* Bajaj 1, J15 and N23 had much higher level of MIC of all the test drugs, except Rf. Surprisingly, step-up mutants of all these 3 strains failed to develop resistance to Rf as well. On the contrary, resistance level of Eb decreased in step-two mutants of *M.tuberculosis* N23. Similar decrease in MIC of Sm was noted in *M.avium* 724 (Table 2). The Pz-resistant step-three mutants of *M.marinum* 50 and *M.intracellulare* 1406 were resistant to Pz at very high levels and increase in MIC values of Eb in these mutants was also significantly high. The resistances of wild-type and mutant mycobacteria differed in most instances by sufficiently large margins (within the limits of confidence of the test system), and therefore appeared to be truly reflective of the actual resistance of such mycobacteria. It is unlikely that the low levels of resistances exhibited by some of the mutants were due to their reduced growth rate, incubation period was kept for sufficiently long period even for slow-growers and these resistances did not seem to depend on drug-modifying enzymes; these, therefore, should not affect their MIC determination. Indeed MICs of several test drugs were found to be elevated simultaneously. Thus these observations on cross-resistance seem to be more easily explainable on the basis of a non-specific reduction in cell membrane permeability of the various Md and Pz mutants in varying degrees and selectivity with respects to different test drugs. A literature survey on the MICs of different antibiotics shows that there had been a significant rise in the highest values that characterize drug resistant strains as well as the lowest values of MICs of drugs/antibiotics (characterizing sensitive strains) with respect to almost all groups of pathogenic bacteria during the last five decades since the beginning of antibiotic era (**Garrod and O' Grady, 1971; Ray et al., 1980**). Development of such resistances has been ascribed to "intrinsic resistance" which is an evolutionary ancient phenotype and can be defined as resistance of any bacterial species to a particular drug/antibiotic that has not been acquired as a result of exposure to such agents (**Fajardo et al., 2008**). Intrinsic resistance is the result of reduced permeability of bacterial envelope and the activity of efflux pumps (**Nikaido and Zgurskaya, 1999**). This suggests that the main physiological role of the components of intrinsic resistance involves the prevention of toxic components by restricting the permeability of the cell or the active export of toxic compounds. However, intrinsic resistance of *M.tuberculosis* has been traditionally attributed to the unusual structure of its mycolic acid containing cell wall that contributes to the low permeability for antitubercular antibiotics and chemotherapeutics (**Jarker and Nikaido, 1994**). The role of efflux mechanisms has been recognized as an important factor in the natural resistances of mycobacteria against tetracyclines, aminoglycosides and fluoroquinolones (**De Rossi et al., 2006**). Even though mutations in several genes are evidently related to drug resistance in *M.tuberculosis* a large number of clinical isolates do not seem to present such classical mutations (**Almeida Da Silva and Palomino, 2011**). It is known that inadvertent and extensive use of antitubercular drugs in tuberculosis patients have continuously contributed to substantial increase in drug resistances in *M.tuberculosis* resulting in emergence of multi-drug resistant and extensively drug resistant strains.

In view of our data and other findings it may be stated that the overall elevation of MIC of antitubercular drugs could also be due to a prolonged and massive use of a vast number chemotherapeutics, many of which have undetected or unsuspected antimicrobial/antitubercular activities. A few among such drugs are several phenothiazines including Md and Pz (**Molnar et al., 1977; Kristiansen and Vergemann, 1986; Ratnakar and Murthy, 1993; Chakrabarty et al., 1993; Dutta et al., 2009; Crowle et al., 1992; Amaral et al.,1996; van Ingen et al., 2009; Advani et al., 2012; Kristiansen et al., 2015**). It may be plausible to presume that many or all of these drugs could have selected mutants resistant to themselves and non-specifically to other antitubercular drugs all at low levels, at the therapeutic dosages at which they are used, thus contributing towards a gradual rise of the baseline of MIC of antitubercular agents. The present study provides experimental evidences for one of such possibilities.

CONCLUSION

The accelerated speed with which tuberculosis has spread in all communities throughout the world and the higher frequency of infection by multidrug and extensively drug resistant *M. tuberculosis* have resulted in an alarming situation. In this study it was observed that many strains of *M.tuberculosis* could be inhibited by both the phenothiazines methdilazine and promethazine.Thus these findings open up a new arena of treatment for multidrug and extensively drug resistant mycobacteria. It may be pointed out here that a structurally similar phenothiazine, thioridazine, has been repeatedly proved to be a potent antitubercular drug. Both the two compounds methdilazine and promethazine are routinely used as antihistaminic drugs while thioridazine is an effective antipsychotic drug. It needs to be pointed out here that since both methdilazine and promethazine are given to patients as antihistaminics and not as neuroleptics development of track resistance is not possible on the basis of prescription. Since the structurally similar neuroleptic drug thioridazine has repeatedly shown to be active against *M.tuberculosis* all such phenothiazines may be grouped together to define the results as a " class mechanism". When the antihistaminic compounds methdilazine and promethazine were allowed to produce mutants resistant to either of them at higher levels, the organisms showed elevated levels of MIC of tested antitubercular drugs. Such a phenomenon may be attributed as intrinsic resistance, which is the result of reduced permeability of bacterial envelope alongwith action of efflux pumps,which, in turn, result in prevention of toxic components into the cell. Therefore, it may be plausible that prolonged and indiscriminate use of such phenothiazines having antitubercular action, often unknowingly, may have allowed development of mutants resistant to themselves and simultaneously cross-resistances to many routinely used antitubercular drugs.

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RAPID AND SIMPLE DETERMINATION OF MINIMUM BIOFILM ERADICATION CONCENTRATION BY A COLORIMETRIC MICROBIAL VIABILITY ASSAY BASED ON REDUCTION OF A WATER-TETRAZOLIUM SALT AND COMBINATED EFFECT OF ANTIBIOTICS AGAINST MICROBIAL BIOFILM

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ABSTRACT

Rapid and simple method for the determination of minimum biofilm eradication concentration (MBEC) using a colorimetric microbial viability assay based on reduction of a tetrazolium salt WST-8 and the biofilm formation method on 96-pegs on the lid of a microtiter plate was developed. The biofilms formed on the 96 pegs were challenged by antibiotics, and the MBEC was then determined from the microbial viability of the biofilms formed on the 96 pegs, assessed by the WST-8 colorimetric assay. The MBECs obtained by the proposed and conventional methods favorably agreed. The most effective inhibitors of *S. aureus* and *P. aeruginosa* biofilms were vancomycin and ciprofloxacin, respectively. In addition, we clarified that *Staphylococcus aureus* biofilm was maximally suppressed by a combination of vancomycin, daptomycin, and teicoplanin. The proposed method yields similar performance to conventional methods, but is faster and more easily implemented. Therefore, the proposed method alleviates the tediousness and time-consuming nature of conventional biofilm susceptibility assay.

Keywords: Biofilm, minimum biofilm eradication concentration, susceptibility testing, antibiotics, tetrazolium salt

INTRODUCTION

Biofilms are formed when microorganisms aggregate with each other or adhere to a solid surface and then encase themselves in a self-produced matrix of extracellular polysaccharides and proteins. Once formed, biofilms are extremely difficult to eradicate and their cellular constituents develop increased antimicrobial resistance. Biofilm formation is thought to be directly attributable to various chronic or device-related infections (**Bhinu 2005; Bridiera *et al.*, 2011; Burmølle *et al.*, 2010**). Therefore, rapid antimicrobial susceptibility testing of microbial biofilm is increasingly important for appropriate patient management and clinical surveillance.

Biofilm-associated infections are treated with different concentrations of antibiotics. To evaluate biofilm resistance to various antibiotics, the biofilm minimum inhibitory concentrations (MICs), broth recovery-based biofilm minimum bactericidal concentrations (MBCs), and minimum biofilm eradication concentrations (MBECs) of the biofilms are assayed (**Qu *et al.*, 2010**). The biofilm MIC is the concentration that inhibits the immediate release of planktonic microbes from biofilms, and is administered during the acute early stage of biofilm-related infections (**Ceri *et al.*, 2001**). The biofilm MBC is the concentration that kills 99.9% of the bacterial cells in the biofilm or sterilizes most of them, and targets chronic biofilm infections (**Aaron *et al.*, 2002; Zhang and Mah, 2008**). The MBEC is the concentration that definitely eradicates all cells in the biofilm, and is administered for successful treatment of biofilm-related infections (**Ceri *et al.*, 2001**). However, a general procedure for the determination of these biofilm parameters is extremely complicated because it is difficult to wash the biofilms cultivated directly on the bottom of wells in microtiter plates and to collect the supernatant on the biofilms with pipetting (**Nett *et al.*, 2011; Qu *et al.*, 2010**). In addition, this method is prone to contamination.

Alternatively, biofilms can be formed on the 96 pegs on the lid of a microtiter plate (**Harrison *et al.*, 2005, 2010**). From an evaluation perspective, this approach offers several advantages over the conventional cultivation technique. First, the 96 peg lids coated with biofilms are easily shifted from one microtiter plate to another, enabling an easier wash process. Second, this method avoids accidental disruption of the biofilm by pipetting. However, to determine the MBEC, we need to count the number of bacteria in the established biofilms. For this purpose, we must detach the bacteria from the biofilms established on the 96 pegs and incubate them on agar medium for 24 h.

Previously, we developed a method for rapid susceptibility testing based on the reduction of a tetrazolium salt (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, or monosodium salt (WST-8)) as an indicator reagent (**Tsukatani *et al.*, 2009, 2012**). In this method, the microorganisms reduce the electron mediator 2-methyl-1,4-naphthoquinone (NQ) to naphthohydroquinone, which in turn reduces WST-8 to formazan. The final reduction product, formazan, maximally absorbs at 460 nm.

This study aims to develop a rapid and simple method for the determination of the MBEC. In our colorimetric microbial viability assay, biofilms are formed on 96 pegs on the lid of a microtiter plate, and the MBEC is evaluated from the reduction of WST-8 to formazan. The advantages of the proposed method are demonstrated by comparison with conventional methods. The proposed method provides a rapid and simple determination of the MBEC. In addition, we also applied our approach to the biofilm susceptibility of *Staphylococcus aureus* and *Pseudomonas aeruginosa* exposed to plural antibiotics.

MATERIAL AND METHODS

Biofilm growth

S. aureus and *P. aeruginosa* used in this study were obtained from the Biological Resource Center at the National Institute of Technology and Evaluation (NBRC, Chiba, Japan), and the American Type Culture Collection (ATCC, Rockville MD, USA). For *P. aeruginosa*, 180 µl of bacterial inoculum grown to approximately 10^6 CFU ml^{-1} in Mueller–Hinton broth (MHB) was added to each well of a 96-well microtiter plate. The plate was then covered with a 96 peg lid and incubated for 24 h, during which time biofilms formed on the 96 pegs. Polypropylene pegs (96 pin plate; Stem Co., Tokyo, Japan) was used for *P. aeruginosa* biofilm. For *S. aureus*, 180 µl of bacterial inoculum grown to approximately 10^7 CFU ml^{-1} in MHB was added to each well of a 96-well microtiter plate. The plate was then covered with a 96 peg lid and incubated for 72 h. In the experiments involving 72-h biofilms, the 96 peg lids were first incubated for 24 h, then transferred to 96-well microtiter plates containing fresh MHB, and incubated for another 48 h. Polystyrene pegs (Nunc-Immuno TSP; Thermo Fisher Scientific Inc., *MA,* USA) was used for *S. aureus* biofilm. The

amounts of established biofilm were measured by a crystal violet stain method (**Antunes *et al*., 2010**).

Detection reagents

The mixture of tetrazolium salt WST-8 (Dojindo Laboratories, Kumamoto, Japan) and 2-methyl-1,4-NQ (Sigma Chemicals, St. Louis, MO, USA) was used as the detection reagent. The WST-8 contains sulfonate groups giving them a net negative charge that reduces their ability to move across cell membranes. Thus, it is necessary to employ 2-methyl-1,4-NQ as an electron mediator to facilitate the cellular reduction of tetrazolium salts. WST-8 was dissolved in distilled water at a concentration of 11.1 mM and the solution was sterilized by passing it through a cellulose acetate membrane filter (pore size = 0.2 μm). 2-Methyl-1,4-NQ (Sigma Chemicals, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide to a concentration of 1.0 mM, then mixed with WST-8 solution at a ratio of 1:9. The prepared detection reagent contained 10 mM WST-8 and 0.1 mM 2-methyl-1,4-NQ.

Determination of MBEC

The MBEC was determined as shown in Fig. 1. The 96 peg lids with established biofilms were placed onto 96-well microtiter plates containing sterile PBS and vortexed for 30 s to dislodge any unattached bacteria. After washing three times in sterile PBS, the 96 peg lids were placed onto 96-well microtiter plates containing180 μl of 2-fold serial dilutions of antibiotics (range 1280 to 1 μg ml^{-1}) in Mueller–Hinton broth (MHB). After 20 h challenge at 35 °C, the 96 peg lids were removed and washed three times with sterile PBS, then placed onto new 96-well microtiter plates containing 180 μl recovery broth (MHB) and incubated for a further 24 h at 35 °C. After the recovery incubation, the 96 peg lids were washed three times with sterile PBS, then placed onto new 96-well microtiter plates containing 200 μl of reaction solution (190 μl MHB and 10 μl detection reagent). After incubation at 35°C, the generated formazan was measured at 460 nm with a microplate reader (VersaMax, Molecular Devices Co., Sunnyvale, CA, USA). The MBEC was read as the lowest concentration of antibiotics for which the absorbance change (relative to the blank value without bacteria) was less than 0.1.

Reference MBECs are determined according to the conventional method reported by Harrison et al. (**Harrison *et al.*, 2005, 2010**). The conventional method computes the MBECs from the number of bacteria in the established biofilms. After the recovery incubation described above, the 96 peg lids are placed in another 96-well microtiter plates containing growth recovery broth, and then sonicated in an ultrasonic sonicating bath. Viable cells in the biofilm can be detected by plating onto appropriate medium, or by measuring increased turbidity over time, using a microplate reader. However, some of viable cells in the biofilm established on the 96 peg lids might be left behind for lack of sufficient sonication. Thus, to implement the conventional method, we collected the biofilm matrix by carefully swabbing each peg with a sterile cotton swab. The tip of the cotton swab was then inserted into a tube containing sterile PBS. The tube was vortexed and sonicated to disintegrate the bacterial clumps and to dislodge the bacteria from the tip of the cotton swab. The bacterial suspension was plated onto agar medium and the number of viable bacteria were counted after 24 h incubation. The MBEC was taken as the minimum concentration of antibiotics for which no visible colonies appeared.

RESULTS AND DISCUSSION

Biofilm formation on the 96-pegs

To establish the biofilm on the 96 pegs, we first noted the optimal growth conditions of *S. aureus* and *P. aeruginosa*, such as peg materials, growth medium, and incubation time. *S. aureus* more readily formed biofilms on polystyrene pegs than on polypropylene (data not shown). On the other hand, *P. aeruginosa* established biofilms more effectively on polypropylene pegs. The biofilm formation medium was MHB because this medium is most commonly used in susceptibility testing. *P. aeruginosa* formed biofilms on the 96 pegs within 24 h, *S. aureus* required 72 h. The biofilms established on the 96 pegs under the optimum conditions were tested for the determination of the MBECs as shown in Fig. 1.

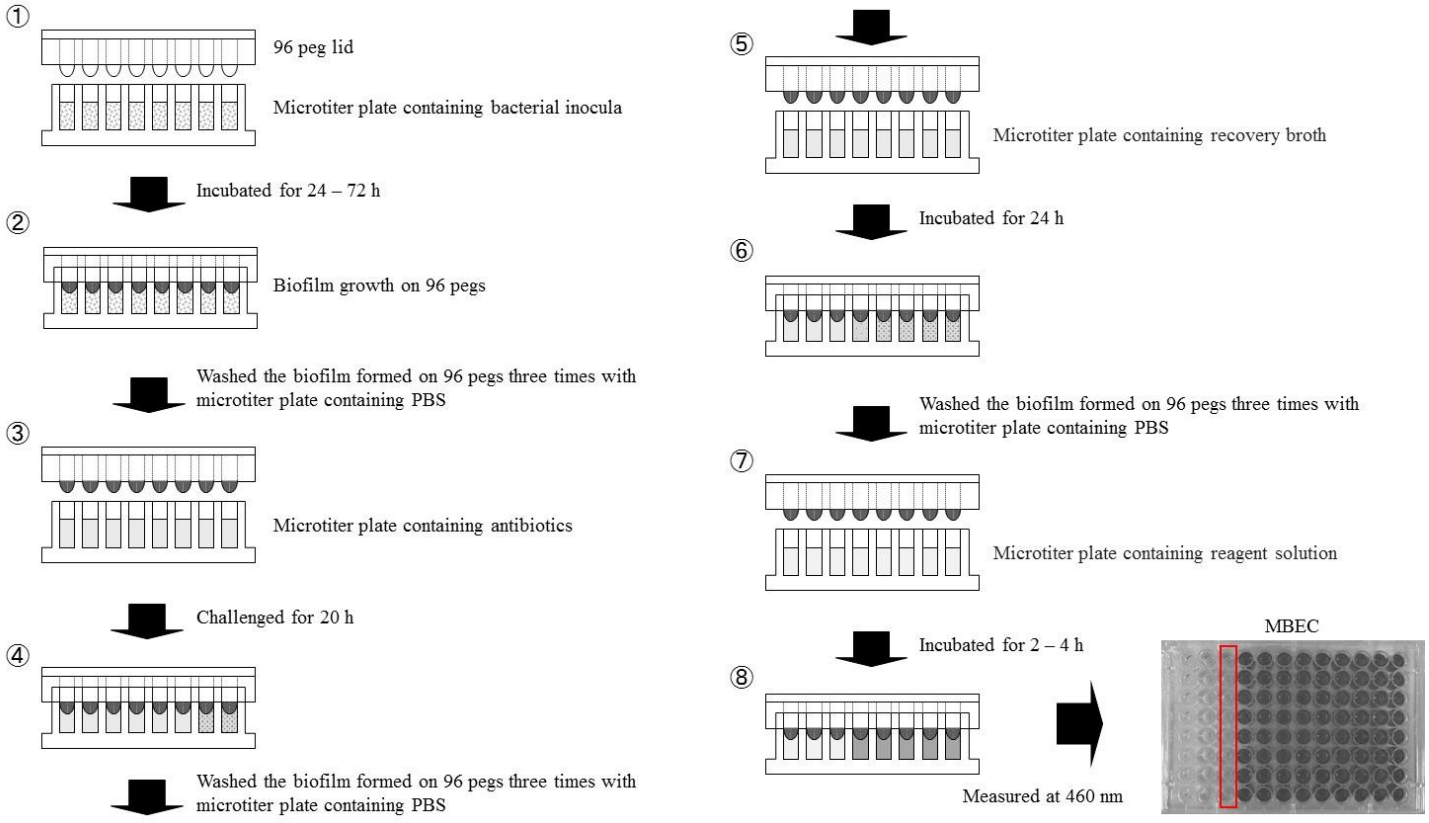

Figure 1 Procedures of biofilm susceptibility tests

Susceptibility of biofilms exposed to a single antibiotic

To evaluate the applicability of the WST-8 colorimetric method to rapid determination of the MBEC, we determined the biofilm susceptibility of *S. aureus* and *P. aeruginosa* in the presence of various single antibiotics by the proposed method.

Figure 2 shows the effects of the WST-8 reduction time on the susceptibility curves of *S. aureus* NBRC13276 and *P. aeruginosa* NBRC13275. For *S. aureus* exposed to vancomycin, the MBEC was estimated as 512 μg/ml at a WST-8 reduction time of 2 h (Fig. 2 (A)). The MBECs obtained at 2 h and 4 h were almost equal. The MBEC for *P. aeruginosa* exposed to ciprofloxacin was estimated as 256 μg/ml at 4 h, but the absorbance at 2 h was relatively low (Fig. 2

(B)). From these results, the WST-8 reduction time was decided as 2 h for *S. aureus* and 4 h for *P. aeruginosa*.

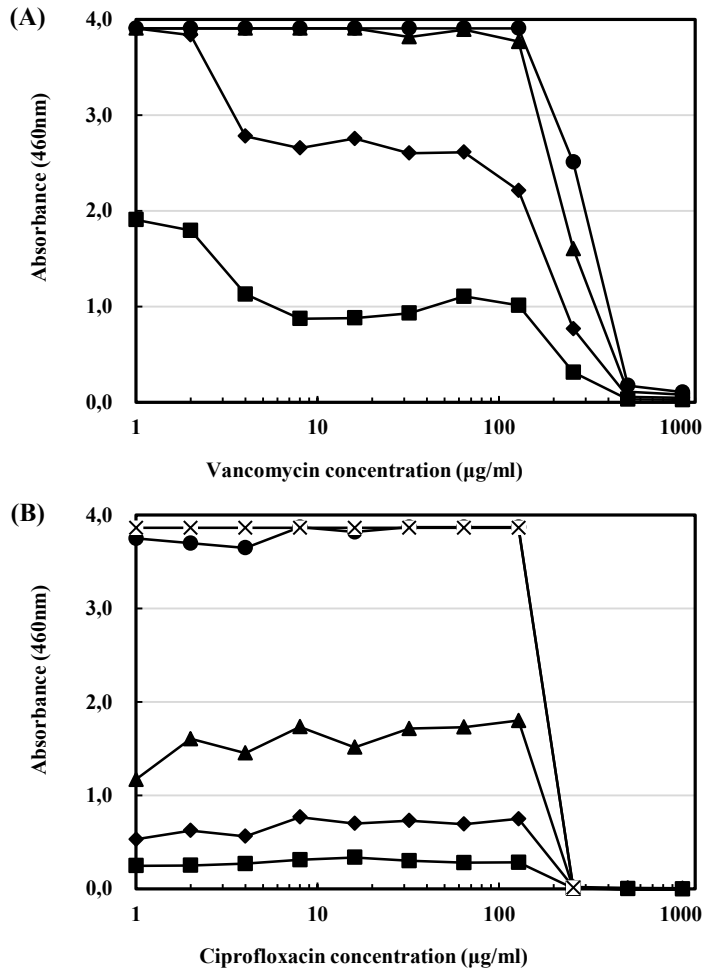

Figure 2 Absorbance (used to determine the MBEC) versus antibiotic concentration for different reduction times of WST-8. Biofilms of S. aureus NBRC13276 (A) and P. aeruginosa NBRC13275 (B).
Incubation time: 1 h, ■; 2 h , ◆; 3 h, ▲; 4 h, ●; 5 h, ×.
The data represent the means for 4 identical wells of a microtiter plate from each experiment.

Tables 1 and 2 show the MBECs of *S. aureus* NBRC13276 and *P. aeruginosa* NBRC13275, respectively, exposed to various antibiotics and evaluated by the proposed and conventional methods. The most effective inhibitors of *S. aureus* and *P. aeruginosa* biofilms were vancomycin and ciprofloxacin, respectively. In addition, reference MBECs were also determined by the conventional method. There was good agreement between the MBECs obtained at 2–4 h using the WST-8 colorimetric method and those obtained after 24 h using the conventional method. In both *S. aureus* and *P. aeruginosa* biofilms, the percentage of the MBEC values located at ±1 $\log_2$ difference was 100%. To better assess the degree of agreement between the MBEC results obtained from the present method and the conventional method, the Wilcoxon signed-ranked test was performed. P values derived from the Wilcoxon signed-rank test demonstrated no significant differences (P = 0.317) between the proposed method and the conventional method.
The conventional method is complicated and the MBEC assays are time consuming because the microbial cells must be first extracted from the biofilm matrix and then incubated for another 24 h (**Harrison *et al.*, 2005, 2010**). On the other hand, the proposed method is simple to implement and yields the MBEC results within a few hours. In addition, the proposed method provides a fast assay of the MBEC. These findings indicate that the proposed method could potentially circumvent the tedious and time-consuming conventional method.

Table 1 MBECs of *Staphylococcus aureus* NBRC13276 biofilms exposed to single antibiotics

Antibiotics	Proposed method	Conventional method
Azithromycin	1024<	1024<
Cefazolin	1024<	1024<
Chloramphenicol	1024<	1024<
Ciprofloxacin	1024<	1024<
Clarithromycin	1024<	1024<
Clindamycin	1024<	1024<
Daptomycin	1024<	1024<
Doxycycline	1024	1024
Erythromycin	1024<	1024<
Gentamicin	1024<	1024<
Imipenem	1024<	1024<
Kanamycin	1024<	1024<
Linezolid	1024<	1024<
Meropenem	1024<	1024<
Minocycline	1024	1024
Oxacillin	1024<	1024<
Phosphomycin	1024<	1024<
Rifampicin	1024<	1024<
Streptomycin	1024<	1024<
Teicoplanin	1024<	1024<
Tigecycline	1024	1024<
Tylosin	1024<	1024<
Vancomycin	512	512

(μg/ml)

Table 2 MBECs of *Pseudomonas aeruginosa* NBRC13275 biofilms exposed to single antibiotics

Antibiotics	Proposed method	Conventional method
Amikacin	1024<	1024<
Azithromycin	1024<	1024<
Aztreonam	1024<	1024<
Ceftazidime	1024<	1024<
Chloramphenicol	1024<	1024<
Ciprofloxacin	256	256
Clarithromycin	1024<	1024<
Colistin	1024<	1024<
Doxycycline	1024<	1024<
Enrofloxacin	1024	1024
Gentamicin	1024<	1024<
Imipenem	1024<	1024<
Kanamycin	1024<	1024<
Levofloxacin	1024	1024
Meropenem	1024<	1024<
Ofloxacin	1024<	1024<
Polymyxin B	1024<	1024<
Streptomycin	1024<	1024<
Tobramycin	1024<	1024<

(μg/ml)

Susceptibility testing of biofilms exposed to plural antibiotics

To investigate the combined effect of antibiotics against microbial biofilm, we applied the proposed method to the biofilm susceptibility of *S. aureus* exposed to combinations of plural antibiotics. Tables 3 and 4 present the MBECs of *S. aureus* in the presence of two and three antibiotics, respectively.
In the two-antibiotic combinations, each antibiotic was compared with vancomycin, the most effective inhibitor of *S. aureus* biofilm in the single-antibiotic study. Vancomycin combined with ciprofloxacin, daptomycin, and teicoplanin effectively reduced the MBEC (Table 3). Therefore, in the three-antibiotic combination, several antibiotics were combined with vancomycin and daptomycin. As shown in Table 4, vancomycin, daptomycin, and teicoplanin most effectively reduced the MBEC of *S. aureus* biofilm. The effectiveness of the vancomycin–daptomycin–teicoplanin combination was also investigated for other *S. aureus* strains. Clearly, biofilms established by other *S. aureus*, including methicillin-resistant *S. aureus* (MRSA), were largely inhibited by this drug combination. In the absence of teicoplanin, the combination of vancomycin and

daptomycin hardly inhibited other *S. aureus* with the exception of *S. aureus* NBRC13276 (data not shown). Therefore, it is thought that the addition effect of teicoplanin is very important for *S. aureus* biofilm eradication.

The glycopeptide antibiotics, such as vancomycin and teicoplanin have similar mechanisms of action on bacterial cell wall synthesis. Vancomycin and teicoplanin are both widely used in the treatment of infections caused by Gram-positive bacteria. Teicoplanin has a longer half-life than vancomycin (**Murphy and Pinney, 1995; Wood, 1996**). Thus, vancomycin requires multiple dosing to maintain adequate serum levels. As shown in Table 1, vancomycin alone showed higher biofilm eradication activity than teicoplanin. In the combination of vancomycin and teicoplanin, it is thought that vancomycin acts *S. aureus* biofilms potentially at an early stage, and then teicoplanin mainly affects the biofilms after the effectiveness of vancomycin is attenuated.

Saginur *et al.* (2006) reported that most of the effective antibiotic combinations against *S. aureus* (including MRSA) include rifampicin. Rifampicin is especially effective when combined with vancomycin and/or fusidic acid (**Saginur *et. al.*, 2006**). Vancomycin is often combined with a secondary antibiotic, usually rifampicin or gentamicin, for the treatment of serious MRSA infections (**Deresinski, 2009**). We demonsrtrated that a new combination of vancomycin, daptomycin and teicoplanin was most effective against *S. aureus* biofilms in vitro.

In the last decade, the role of biofilms in various chronic bacterial infections has been recognized (**Burmølle *et al.*, 2010**). The findings of the present study suggest the utility of the proposed method as a rapid and simple assay of microbial biofilm. In the future, we hope that our method will be adopted as an alternative to the conventional method for rapid and simple susceptibility testing of microbial biofilm.

Table 3 MBECs of *Staphylococcus aureus* NBRC13276 biofilms exposed to combinations of two antibiotics, determined by the proposed method

Antibiotics	Vancomycin			
	16	32	64	128
Cefazolin	128<	128<	128<	128<
Ciprofloxacin	128	128	128	64
Clarithromycin	128<	128<	128<	128<
Clindamycin	128<	128<	128<	128<
Daptomycin	128<	128<	64	4
Gentamicin	128<	128<	128<	128
Linezolid	128<	128<	128<	128<
Meropenem	128<	128<	128<	128<
Minocycline	128<	128<	128<	128<
Rifampicin	128<	128<	128<	128<
Teicoplanin	128<	128<	2	1
Tigecycline	128<	128<	128<	128<

(μg/ml)

Table 4 MBECs of biofilms of various *Staphylococcus aureus* strains exposed to combinations of three antibiotics, determined by the proposed method

Antibiotics	**Vancomycin / Daptomycin**			
	8/4	16/4	32/4	64/4
S. aureus NBRC13276				
Ciprofloxacin	32<	32<	32<	8
Gentamicin	32<	32<	32	2
Minocycline	32<	32<	32<	32<
Rifampicin	32<	32<	32<	32
Tigecycline	32<	32<	32<	32<
Teicoplanin	8	4	4	1
S. aureus ATCC29213				
Teicoplanin	32<	32<	32<	32
S. aureus NBRC14462				
Teicoplanin	32<	32<	32<	32
S. aureus ATCC43300 (MRSA)				
Teicoplanin	32<	32<	32	16

(μg/ml)

CONCLUSION

We have develop the rapid and simple method for the determination of MBEC using a colorimetric microbial viability assay based on reduction of a tetrazolium salt WST-8 and the biofilm formation method on 96 pegs on the lid of a microtiter plate, and demonstrated the advantages of the proposed method as compared to the conventional methods. There was good agreement between the MBECs obtained at 2–4 h using the WST-8 colorimetric method and those obtained after 24 h using the conventional method. In addition, by the proposed method, we clarified that a combination of vancomycin, daptomycin and teicoplanin was most effective for *Staphylococcus aureus* biofilm. The proposed method yields similar performance to conventional methods, but is faster and more easily implemented. Therefore, the proposed method alleviates the tediousness and time-consuming nature of conventional biofilm susceptibility assay.

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OPTIMAZTION OF GAMMA-DECALACTONE PRODUCTION BY YEAST *YARROWIA LIPOLYTICA* USING THE TAGUCHI METHOD

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ABSTRACT

The γ-decalactone is one of the lactones with peachy aroma which has been approved as food additive by FDA. The aim of this study was to optimize media composition and conditions for microbial biotransformation of ricinoleic acid and castor oil as substrates to γ-decalactone using the yeast *Yarrowia lipolytica*. The *Y. lipolytica* DSM 3286 strain was used as biotransformation agent in different trails designed by Taguchi method. The highest concentration of γ-decalactone was 62.4 and 52.9 mg/L from 1.5% ricinoleic acid and 2.5% castor oil, respectively. Nitrogen sources exhibited significant effect on the biotransformation. The maximum γ-decalactone production occurred at pH 6. The results showed that the composition of biotransformation medium composition is important for γ-decalactone production.

Keywords: γ-Decalactone, *Yarrowia lipolytica*, Ricinoleic acid, Castor oil, Biotransformation, Taguchi method

INTRODUCTION

Lactones are attractive molecules as additive for food and pharmaceutical products due to their pleasant flavor. The γ-decalactone is one of the lactones with peach flavor which has been approved as food additive by FDA. Microbial production of flavors as an alternative route to extraction from plants or chemical synthesis has received great deal of attention. A variety of microorganisms can be used to synthesize flavor compounds using simple nutrients. The main driving force for microbial production of flavor compounds is that the flavor compounds produced by microorganisms can be labeled as "natural" **(Longo & Sanromán, 2006; Scharder, 2007; Schrader, Etschmann, Sell, Hilmer, & Rabenhorst, 2004)**. Lactones are ubiquitous flavor and aroma constituents of many essential oils and plant volatiles (**Başer & Demirci, 2007**). The γ-decalactone, the lactone of 4-hydroxydecanoic acid, is the most widely used flavor lactone exhibiting an oily-peachy aroma. Okui et al. were the first who noticed the accumulation of γ-decalactone during the growth of a *Candida* species on ricinoleic acid (**Okui, Uchiyama, & Mizugaki, 1963; Romero-Guido *et al.*, 2011**).
The medium composition is of great importance in microbial production of γ-decalactone. Microorganisms can use ricinoleic acid as suitable substrate for production of this aroma compound (**Lee & Chou, 1994**). Castor oil which contains 85% of ricinoleic acid could be used as readily available, inexpensive substrate for γ-decalactone production (**Dufosse *et al.*, 1998; Neto, Pastore, & Macedo, 2004**).
Environmental conditions such as temperature and pH are important and influence the γ-decalactone production. The nitrogen sources effect on this fermentation is complex. The highest yields were obtained using complex nitrogen sources which is probably due to supplying trace nutrients and minimizing the toxic effects of fatty acids by sequestering them (**Maume & Cheetham, 1991**).
The non-conventional yeast *Yarrowia lipolytica* have good potential to production of γ-decalactone. In the previous studies, this yeast has been used for γ-decalactone production from ricinoleic acid and castor oil as substrates (**Aguedo, Wache, Belin, & Teixeira, 2005; N. Gomes, Teixeira, & Belo, 2010; Moradi, Asadollahi, & Nahvi, 2013**).
Since control of bioconversion parameters was shown to significantly affect γ-decalactone production, a large number of experiments are needed to optimize bioconversion conditions (**Lee & Chou, 1994**). To circumvent this problem, Taguchi method was used for optimization of bioconversion conditions. Taguchi method requires only a small number of experiments to study the entire parameters involved in process. Furthermore, this method allows studying effects of multiple factors on the process yield in a fast and economic way using an orthogonal array design (**Montgomery, 1991**). Taguchi method shows the importance of distinct values to improve the process and product quality (**Fraley, Oom, Terrien, & Alewsk, 2007**). This study therefore aimed to optimize important factors affecting γ-decalactone production from ricinoleic acid and castor oil as substrates by the Taguchi method.

MATERIAL AND METHODS

Microorganism and culture conditions

Y. lipolytica DSM 3286 was cultured on YPD medium at 29 ˚C, and maintained at 4 ˚C on YPD-agar (**Barth & Gaillardin, 1996**). Basal main medium for γ-decalactone contained ricinoleic acid or castor oil, yeast extract, peptone. The flasks were incubated in shaker-incubator at 200 rpm and 29 ˚C (**Moradi *et al.*, 2013**).

Design of experiments

The Taguchi fractional factorial experiment design approach has been used for optimization of production variables. It is a robust methodology against uncontrollable environmental changes (**Patil, Sachin, Wakte, & Shinde, 2014**). The Qualitek-4 software was used for Taguchi experimental design in this research. In the previous study, it was shown that substrate (ricinoleic acid or castor oil), yeast extract concentration, peptone concentration, and pH are the four main factors affecting production of γ-decalactone by *Y. lipolytica* (**Moradi *et al.*, 2013**). Therefore, these four main factors were used each at four levels as shown in Tables 1-3.

Lactone extraction and detection

For extraction and analysis of γ-decalactone, 50 mg/L of γ-valerolactone (internal standard) was added to 2 mL of filtered sample of the culture medium (**Alchihab *et al.*, 2009**). Afterwards, diethyl ether was used as organic phase to lactone extraction (**Aguedo, Wache, Coste, Husson, & Belin, 2004; Groguenin *et al.*, 2004**). The ether layer was recovered and the analysis was performed with HP6890 gas chromatograph (Agilent) equipped to an FID detector. The analytes were separated on a HP-5 capillary column with helium as carrier gas at a flow

rate of 3 mL/min. A split/splitless injector was used in the split mode (split ratio 1:30). The injector and FID temperatures were 200 and 250 ˚C, respectively. The oven temperature was increased from 60 to 195 ˚C at a rate of 20 ˚C /min and then at a rate of 10 ˚C /min to 270 ˚C (**An, Joo, & Oh, 2013; Moradi *et al.*, 2013)**.

RESULTS

In order to investigate the best conditions for optimum γ-decalactone production from ricinoleic acid or castor oil as substrates by *Y. lipolytica* DSM 3286, trails were done according to experiments proposed by the Qualitek-4 software (Fig. 1 and Fig. 2).

In the case of ricinoleic acid as substrate, maximum γ-decalactone production of 62.4 mg/L was obtained at concentrations of ricinoleic acid 15 mL/L, peptone 3 g/L, and yeast extract 9 g/L at pH 6 (Trail 10).

The main effects of each factor on γ-decalactone production from ricinoleic acid by *Y. lipolytica* DSM 3286 were determined by Qualitek-4 software (Fig. 3). Lower γ-decalactone production rate was observed at higher ricinoleic acid concentrations and pH whereas increasing yeast extract concentration had positive effect and enhanced production rate of γ-decalactone. Peptone concentration had variable effects on γ-decalactone production.

Optimum conditions for γ-decalactone production from castor oil were obtained as 25 mL/L of castor oil, 9 g/L of peptone and yeast extract, and pH 6 in which 52.9 mg/L of γ-decalactone was produced. However, this combination had not been used in any of the experiments designed by the software.

The main effects of each factor on γ-decalactone production from castor oil by *Y. lipolytica* DSM 3286 were determined by Qualitek-4 software (Fig. 4). If increase the amounts of castor oil and yeast extract, the production of γ-decalactone will be increased. Peptone had no significant effect on γ-decalactone production. Acidic pH is better than neutral pH for production of γ-decalactone by *Y. lipolytica* DSM 3286 (Fig. 4).

Since maximum γ-decalactone titer was observed at the highest concentration of castor oil (25 mL/L), the effect of higher concentrations of castor oil on the γ-decalactone production was examined. However, it was found that higher concentrations of castor oil did not further improve γ-decalactone production (Fig. 5).

Table 1 Levels of factors for ricinoleic acid as substrate

Factors	Level 1	Level 2	Level 3	Level 4
Ricinoleic acid (mL/L)	5	10	15	20
Peptone (g/L)	0	3	6	9
Yeast extract (g/L)	0	3	6	9
pH	4	5	6	7

Table 2 Levels of factors for castor oil as substrate

Factors	Level 1	Level 2	Level 3	Level 4
Castor oil (mL/L)	10	15	20	25
Peptone (g/L)	0	3	6	9
Yeast extract (g/L)	0	3	6	9
pH	4	5	6	7

Table 3 The L16 orthogonal array for trails

Trial number	Ricinoleic acid or castor oil (mL/L)	Peptone (g/L)	Yeast extract (g/L)	pH
1	Level 1	Level 1	Level 1	Level 1
2	Level 1	Level 2	Level 2	Level 2
3	Level 1	Level 3	Level 3	Level 3
4	Level 1	Level 4	Level 4	Level 4
5	Level 2	Level 1	Level 2	Level 3
6	Level 2	Level 2	Level 1	Level 4
7	Level 2	Level 3	Level 4	Level 1
8	Level 2	Level 4	Level 3	Level 2
9	Level 3	Level 1	Level 3	Level 4
10	Level 3	Level 2	Level 4	Level 3
11	Level 3	Level 3	Level 1	Level 2
12	Level 3	Level 4	Level 2	Level 1
13	Level 4	Level 1	Level 4	Level 2
14	Level 4	Level 2	Level 3	Level 1
15	Level 4	Level 3	Level 2	Level 4
16	Level 4	Level 4	Level 1	Level 3

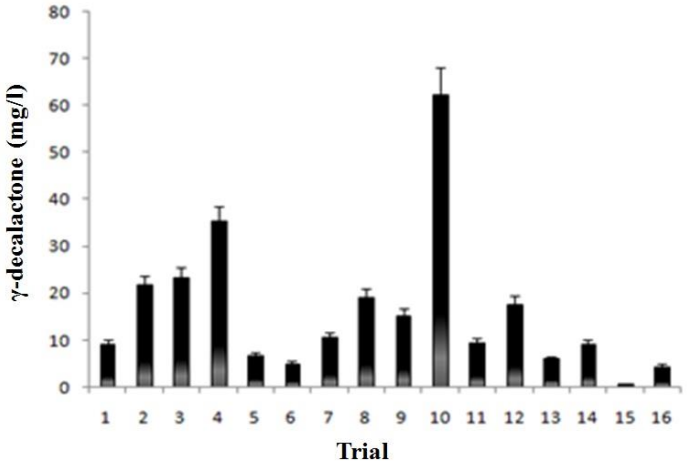

Figure 1 The results of different trails proposed by Qualitek-4 software for γ-dacalactone production on ricinoleic acid as substrate by *Y. lipolytica* DSM 3286.

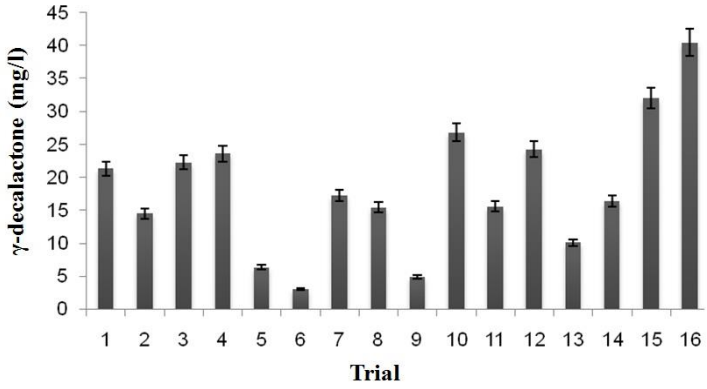

Figure 2 The results of different trails proposed by Qualitek-4 software for γ-dacalactone production on castor oil as substrate by *Y. lipolytica* DSM 3286.

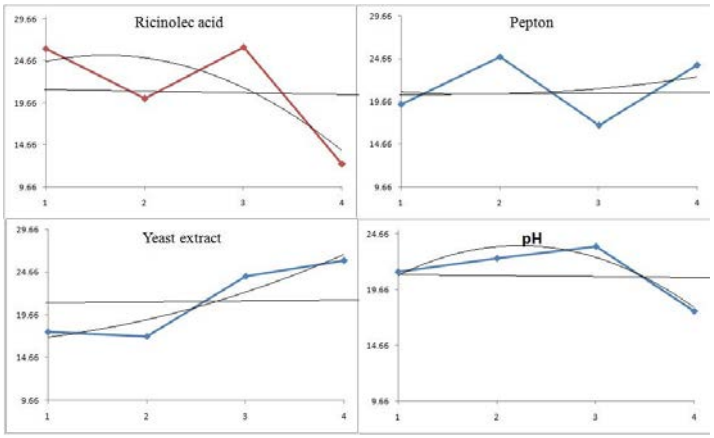

Figure 3 The main effects of each factor on γ-decalactone production from ricinoleic acid by *Y. lipolytica* DSM 3286.

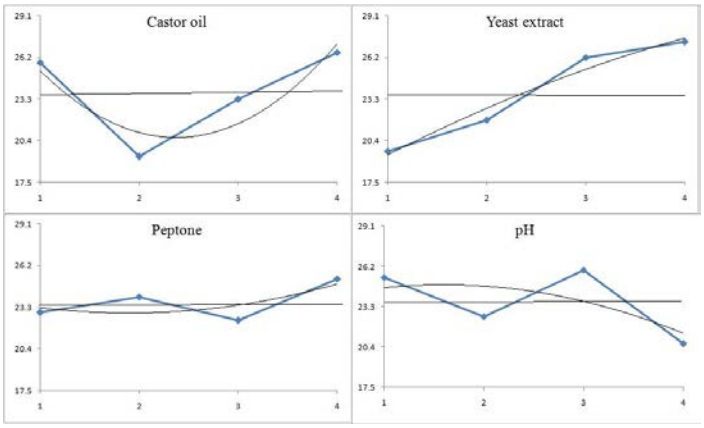

Figure 4 The main effects of each factor on γ-decalactone production from castor oil by *Y. lipolytica* DSM 3286.

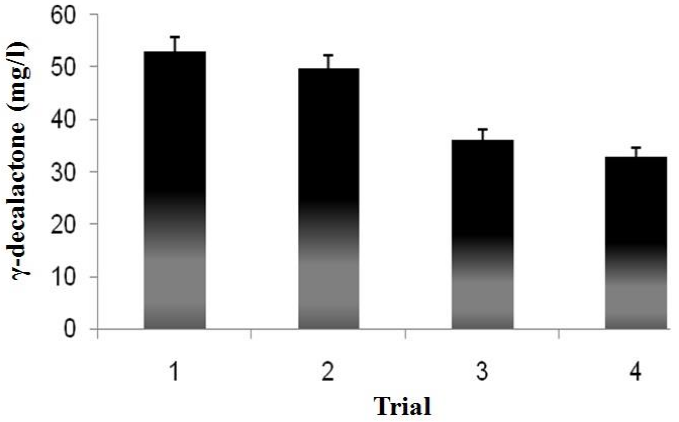

Figure 5 Confirmatory tests for γ-decalactone production from castor oil by *Y. lipolytica* DSM 3286. Trail (1) is a confirmatory test for proposed experiment by the software; Trails (2,3,4) contain 30, 35 and 40 mL/L of castor oil, respectively, along with other suggested factors for optimization including peptone 9 g/L, yeast extract 9 g/L and pH 6.

DISCUSSION

Yeasts are excellent biocatalysts in the field of alkane and fatty acids transformation into dicarboxylic acids and lactones (**Waché, 2013**). The γ-decalactone production can be improved by cell density, oil concentration and oxygen transfer rate in batch and step-wise fed-batch cultures of *Y. lipolytica* (**Braga & Belo, 2015**).

Some of yeasts such as *Y. lipolytica* can produce γ-decalactone from ricinoleic acid as key precursor and substrate **(Darvishi Harzevili, 2014**). Castor oil is natural and nontoxic oil, biodegradable, and a renewable resource obtained from the seeds of the castor plant *Ricinus communis* (**Puthli, Rathod, & Pandit, 2006**). The castor oil is rich in ricinoleic acid and therefore could be used as cheap source for γ-decalactone production (**Braga & Belo, 2013; Nelma Gomes, Braga, Teixeira, & Belo, 2013; N. Gomes *et al.*, 2010**). *Y. lipolytica* is able to hydrolyze castor oil to ricinoleic acid and gradually use it, because a high concentration of this acid has inhibitory effect on the growth of yeast cells. Presence of castor oil at the beginning of the fermentation and then adding it at a later stage could enhance the γ-decalactone production level by *Y. lipolytica* because the production of γ-decalactone needs continuous induction of the β-oxidation pathways or fatty acid uptake and export systems (**Feron, Blin-Perrin, Krasniewski, Mauvais, & Lherminier, 2005; Maume & Cheetham, 1991**). Yeast extract and peptone were used as nitrogen sources for the production of γ-decalactone in this research according to previous studies (**Alchihab *et al.*, 2009; Moradi *et al.*, 2013**).

Four factors with four levels were chosen for optimization of γ-decalactone production by the Taguchi method as fractional factorial experiment design. First factor was carbon source (ricinoleic acid or castor oil) which have been investigated as substrate and an activator for enzymes in the pathway of γ-decalactone synthesis in *Y. lipolytica*. Second and third factors were nitrogen sources including yeast extract and peptone that are important to increase cell growth and biotransformation. Last factor was pH which should be optimized to support maximum cell growth and γ-decalactone production.

Maximum production of γ-decalactone was 62.4 and 52.9 mg/L by *Y. lipolytica* DSM 3286 on ricinoleic acid and castor oil as substrates, respectively. According to the previous studies, the nitrogen source effect was complex on this bioconversion. The γ-decalactone yield was low in the media with less than 2 g/L nitrogen content. Furthermore, a little additional γ-decalactone was produced in media containing greater than 20 g/L of nitrogen concentrations. The function of these complex protein sources is important to increase cell growth and minimize toxic effect in biotransformation (**Maume & Cheetham, 1991; Patil *et al.*, 2014**).

The optimum pH was 6 to γ-decalactone production for both substrates. The bioconversion of ricinoleic acid into 4-HDA and then γ-decalactone was obtained at acid pH (**Lee, Lin, & Chou, 1995**). Other researchers used this pH (**Aguedo, Ly, et al., 2004; Alchihab *et al.*, 2009; Wache, Aguedo, LeDall, Nicaud, & Belin, 2002; Wache, Aguedo, Nicaud, & Belin, 2003**) but in this work pH was optimized step by step from acidic to neutral.

CONCLUSION

In conclusion, biotransformation medium composition is important for γ-decalactone production. In particular, interaction of carbon sources and yeast extract as nitrogen source is important. Furthermore, nitrogen sources are important to increase cell growth and biotransformation. Microbial fermentation was used as a potential tool to the production of natural lactones and attractive subject for researchers in this filed. The production of lactone will be established on the industrial scale because it is an extracellular product and can be produced easily with higher yields by culturing yeast in a bioreactor.

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OPTIMIZATION OF THERMOSTABLE ALPHA-AMYLASE PRODUCTION FROM *GEOBACILLUS* SP. D413

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ABSTRACT

The qualitative and quantitative α-amylase production capacities of six thermophilic bacilli were screened. *Geobacillus* sp. D413 was selected for enzyme optimization, as it displayed higher α-amylase activity. The maximum enzyme activities of D413 and *G. stearothermophilus* ATCC 12980^T were observed at the time of 72 h. While the optimal pH of medium for bacterial growth and enzyme production of D413 (pH 7.0) differed from ATCC 12980^T (pH 8.0), the optimal temperature for enzyme production was 55°C for both. The effects of various carbon and nitrogen sources were determined by changing their concentrations. The highest bacterial growth and enzyme production were sustained by the starch and maltose containing medium. Both bacterial growth and enzyme production were inhibited by NH_4Cl. D413 and ATCC 12980^T amylases showed optimal activity at 65ºC, pH 9.0 and at 65ºC, pH 7.5, respectively. They remained active over temperature and pH ranges of 45-75ºC and 4.0-10.5. Their activities retained 65% and 54% when incubated at 75ºC for 10 min and 98-86.5% and 95-84.5% at pH 4.0-10.5 for 15 h at 37ºC. In conclusion, the α-amylase production conditions of D413 have been optimized which can be useful in biotechnological processes such as hydrolysis of starch to glucose.

Keywords: Thermostable, α-Amylase, Production, Optimization, *Geobacillus* sp.

INTRODUCTION

α-Amylases (EC 3.2.1.1, 1,4-α-D-glucan glucanohydrolases and endoamylases) degrade starch hydrolyzing enzymes by breaking internal α-1,4-glucosidic linkages and produce reducing sugars. Starch is an immanent polysaccharide composed of two high-molecular weight components such as amylose and amylopectin. Amylose is a linear chain consisting of units from glucose molecules linked by α-1,4 glycosidic bonds. Amylopectin is a branched molecule containing α-1,6 linking branch points in addition to α-1,4 glycosidic bonds. α-amylases are produced abundantly by plants and microorganisms and produce a wide class of industrial enzymes constituting approximately 30% of the world enzyme market **(Van der Maarel *et al.*, 2002).**
Of those amylases, microbial α-amylases have wide applications in industrial processes such as starch degradation, production of glucose and fructose syrup as well as fruit juices and alcoholic beverages, and also in applications such as detergent, paper and textile industries. Recent studies especially focus on the role of thermostable enzymes in biotechnology and industry, since many industrial enzymatic reactions are performed at elevated temperatures. Therefore, thermostable α-amylases take advantage of usage potential in these processes. The microbial α-amylases have been described and characterized in some species including *Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus licheniformis, Geobacillus thermodenitrificans* HRO10 **(Ezeji and Bahl, 2006)**, *Geobacillus stearothermophilus* ATCC 12016 and *Geobacillus stearothermophilus* ATCC 12980^T **(Underkofler, 1976; Suzuki *et al.*, 1984; Ferner-Ortner-Bleckmann *et al*,. 2009)**. Some amylase producing strains and their pH and thermal stabilities are also listed in Table 1. Here we report the characteristic properties of *Geobacillus* sp D413 α-amylase by presenting the thermal and pH activity and stability of its enzyme as well as the optimal culture conditions for enzyme production.

Table 1 Some source microorganisms and properties of their thermostable or pH stable α-amylases

Organism	Optimum Temperature (°C)	Residual Activity (%)	Optimum pH	Residual Activity (%)	Reference
Bacillus sp. I-3	70	50 (80°C-2.5 h)	7.0	80	**Goyal *et al.*, 2005**
Bacillus sp ANT-6	80	87.7 (100°C-60 min)	10.5	55 (pH:10 15 h)	**Arikan *et al.*, 2003**
Bacillus sp PS-7	60	78 (50°C -6h)	6.5	96 (pH:5.0 90 min)	**Sodhi *et al.*, 2005**
L. manihotivorans	55	70 (50°C-1 h)	5.5	80 (pH:5.5 60 min)	**Aguilar *et al., 2000***
Bacillus KSM-K38	55-60	20 (50°C - 30 min)	8.0-9.5	80 (pH:11 30 min)	**Hagihara *et al.*, 2001**
Bacillus sp. Strain PM1	50	67 (50°C-60min)	8.0	78 (pH:8.0 4h)	**Sharma *et al.*, 2014**
B. amyloliquefaciens P-001	60	73 (50°C -30 min)	6.5	-	**Deb *et al.*, 2013**
G.thermodenitrificans HRO10	75-80	0 (70°C-30 min)	5.5	-	**Ezeji and Bahl, 2006**

MATERIALS AND METHODS

Bacterial isolates

In this study, formerly isolated six thermophilic bacilli and reference strain *G. stearothermophilus* DSMZ 22^T were screened for their qualitative and quantitative amylolytic activities. These thermophilic bacilli were formerly isolated from a polyphasic study and known to be amylase-producing strains **(Coleri *et. al.*, 2009).** Their 16S rRNA gene sequence analyses were also determined from our previous studies and their gene sequences were found in GenBank databases **(Cihan *et al.*, 2011 and 2013).** The thermophilic isolates used in this study were *Geobacillus stearothermophilus* A113 (FJ429596), *Geobacillus* sp. C304 (FJ429574) and *Geobacillus* sp. D413 (FJ430040), *Anoxybacillus caldiproteolyticus* D504 (FJ430047), and *Anoxybacillus caldiproteolyticus* D621 (FJ430050). The reference strain *Geobacillus stearothermophilus* ATCC 12980^T was also used for comparison purposes.

Qualitative and quantitative amylase screening assays

In qualitative screening assay, a modified method of **Dheeran *et al.* (2010)** medium was used for bacterial growth. Amylolytic activity was tested on this modified Dheeran medium containing 0.5% peptone, 0.5% yeast extract, 0.5% NaCl, 1.0% soluble starch and 3.0% agar (pH 7.0) after incubation for 24 h at 55°C. Then the plates were treated with 0.2% I_2 in 2% KI solution and isolates having starch digestion zones around their colonies were determined as amylolytic **(Coleri *et al.*, 2009).** The halo zone diameters were measured (cm) in order to compare the amylase producing strains, which were produced due to hydrolysis of starch.

When determining the quantitative amylolytic activity for the screening assay, a modified **Santos and Martins (2003)** medium (1.0% tryptone, 0.5% yeast extract, 1.0% soluble starch) was used for enzyme production. The pH of the medium was adjusted according to the bacteria. The growing cells were suspended in 0.85% sterile NaCl and adjusted to 0.2-0.4 absorbance at 660 nm, and then 500 µL from this suspension was inoculated into 5 mL of enzyme production medium and incubated at 55 °C by shaking at 150 rpm for 72 h. The cells were collected by centrifugation at 5000 rpm for 15 min at 4 °C, and the wet weights of the cells were measured. α-Amylase production capacities of the isolates were designated based on their total enzyme amount per cell-yield (U/g). All the experiments were at least triplicate.

Determination of optimal culturing conditions for enzyme production

In order to optimize the growth pH, temperature and time for achieving the maximum enzyme production, the modified Santos and Martins broth (1.0% tryptone, 0.5% yeast extract, 1.0% soluble starch,) was used when cultivating the bacteria before measuring the amylase activities. The supernatant was obtained as explained before in the quantitative amylolytic activity assay and was used as an enzyme source for further experiments. In order to compare the α-amylase production capacities of the bacteria, total enzyme activity values were divided into biomass of bacterial pellet wet weight (U/g) throughout all the experiments in this study.

When determining the optimal cultivation conditions for amylase production, either the incubation temperature or the pH of the modified Santos and Martins medium was changed. For the determination of thermal conditions on enzyme production, bacterial isolates were incubated in this medium by changing the incubation temperature from 50 to 65°C. On the other hand, the pH of the medium was adjusted to various pH values between 3.0 and 10.0 in order to determine the effect of pH on amylase production. In addition, a time-course was carried out during 96 h in this broth medium by taking samples at 24 h intervals. The pH changes in the medium and also the cell weights were also measured during 96 h cultivation. All the triplicate experiments were further taken into spectrophotometric amylase assay to measure their enzyme production.

Enzyme assay

The α-amylase activity was determined by spectrophotometric method with measuring the hydrolysis of soluble starch as substrate. The standard reaction mixture was prepared by adding appropriately diluted 0.5 ml enzyme solution to 0.5 ml 0.2 M sodium phosphate buffer with 0.5 ml 2% soluble starch. Reaction was carried out at 65°C for 10 min and it was stopped by boiling 5 min after addition of 1 ml DNS. When the reaction tube was cooled, the amylase activity was measured spectrophotometrically at 540 nm. One unit of α amylase activity was defined as the amount of enzyme that catalyzed the liberation of reducing sugar equivalent to 1 µmol of maltose per min under the assay conditions. The millimolar extinction coefficient of maltose at 540 nm, pH 7.0 and at 65°C were measured as 1.454 $M^{-1}.cm^{-1}$ and used to calculate the amount of product yielded. The enzyme assays were performed at least three times.

Determination of the effect of different carbon and nitrogen sources on amylase production

Various carbon sources were used for the determination of their effects on amylase production. Therefore, thermophilic isolates were grown in different modified Santos and Martins medium containing 1 % carbon sources such as sucrose, lactose, dextrose, maltose, and soluble starch (**Kıran *et al.*, 2005, Pavithra *et al.*, 2014**). Of those sources, the concentration of starch was also screened in wider ranges from 0.2 to1 %. Moreover, different nitrogen sources including tryptone, yeast and ammonium chloride, in concentrations from 0.2 to 1%, were studied for their effect of enzyme production. In these assays, the pH and temperature were adjusted to the determined optimal value according to the bacteria or their enzymes used for both growth conditions and for enzyme assays.

Determination of the effect of temperature and pH on α-amylase activity and stability

The effect of temperature on enzyme activities was determined on the crude enzymes between 45°C to 75°C with 5°C intervals. For stability tests, enzymes were heated for 10 min at different temperatures, then quickly chilled on ice and assayed for the remaining activity at its optimal pH and temperature. The effect of pH on the enzyme activity was determined using 0.02 M sodium-citrate (pH 4.0, 4.5, 5, 5.5, 6.0), 0.1 M sodium phosphate (pH 6.5, 7, 7.5, 8.0) and 50mM glycine-NaOH (pH 8.5, 9.0, 9.5, 10.0, 10.5) buffers at 65°C, all instead of 0,2 M potassium phosphate buffer in the standard assay mixture. The effect of pH on α-amylase stability was examined by incubating the enzymes in the same buffers (pH 4.0-10.5) at 37°C for 15 h as reported in the activity test. The activity of the not-heated and not pH-treated enzyme was expressed as 100 %.

RESULTS

Screening results and optimization of culture conditions

In this study, six thermophilic bacilli and an amylase producing reference strain were screened for their α-amylase activity both qualitatively and quantitatively. The zone diameters and the specific α-amylase activity results obtained from screening assays were listed in Table 2. According to these results, the highest α-amylase producing strain was found as *Geobacillus* sp. D413 with a zone diameter of 0.5 cm and with a specific enzyme activity of 15.84 U/g, whereas these values were determined as 15.79 U/g and 0.3 cm for the reference strain *G. stearothermophilus* ATCC 12980^T. Consequently, as the the largest zone diameter after 24 h and the maximum specific activity after 72 h were observed in *Geobacillus* sp. D413, D413 isolate and the reference strain were selected for further optimization analyses.

Table 2 The zone diameters and specific enzyme activities of bacteria obtained from screening assays

Bacteria	Zone diameters (cm)	Enzyme activity/biomass (U/g)
Geobacillus sp. D413	0.5	15.84
Geobacillus stearothermophilus ATCC 12980^T	0.3	15.79
Geobacillus sp. C304	0.4	13.33
Geobacillus stearothermophilus A113	0.2	9.32
Anoxybacillus caldiproteolyticus D504	0.1	8.46
Anoxybacillus caldiproteolyticus D621	0.2	7.76
Anoxybacillus caldiproteolyticus D623	0.2	6.18

When the culture conditions were adjusted between the temperatures from 55-65°C (Fig. 1a) and pH values of 3.0-10.0 (Fig. 1b), the optimal incubation temperature for the maximum amylase production was determined as 55 ^{0}C for both of the bacteria, but they differed in optimal bacterial growth pH for the highest amylase production. pH 7.0 (12,9 U/g) and pH 8.0 (11,73 U/g) were found as optimal values for the isolate D413 and strain ATCC 12980^T, respectively. If the culture medium was adjusted to a pH value of 4.0, it seems that *Geobacillus* sp. D413 could not grow in the medium. This case was shown at previous studies. **(Dheeran *et al.*, 2010; Suman and Ramesh, 2010)**. Furthermore, enzyme production was diminished, when the temperature was below and above 55°C.

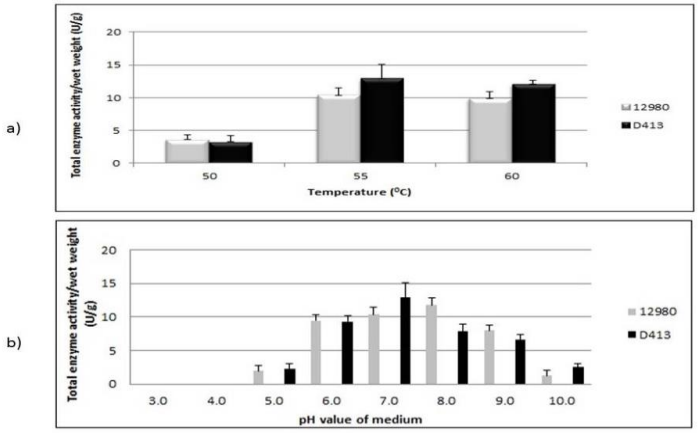

Figure 1 Enzyme production of *G.stearothermophilus* ATCC 12980^T and *Geobacillus* sp. D413 in medium containing 1 % starch, 0.5 % yeast extract and 1 % tryptone at different a) temperatures, and b) pH.

In addition, *Geobacillus* sp. D413 isolate and standard strain were incubated at 55°C and at 150 rpm (pH 7.0 or 8.0) for 96 h in broth medium containing 1% starch (Fig. 2). The experiments on growth patterns and time-course of α-amylase production by D413 and ATCC 12980^T revealed that the maximum extracellular enzyme activity was observed at the time of 72 h, for both D413(15.84 U/g) and ATCC 12980^T (15.79 U/g). Moreover, time-course revealed that the highest α-amylase production was achieved when the pH of the medium were diminished up to 5.0 on 72 h from their optimal pH values (7.0 or 8.0) for both of the strains. Therefore, in order to reach to the highest amylase production, both D413 and ATCC 12980^T were incubated at 55°C and pH 7.0 or 8.0 by shaking at 150 rpm, in rest of the studies, respectively.

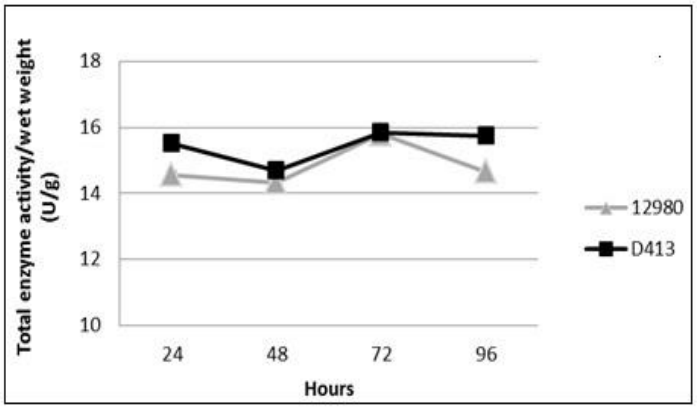

Figure 2 Time course of *G.stearothermophilus* ATCC 12980^T (Δ) and *Geobacillus* sp. D413 (□) during 96 h in medium containing 1 % starch, 0.5 % yeast extract and 1% tryptone at 55°C.

Effects of different carbon sources on enzyme activities

Among the different carbon sources such as starch, maltose, sucrose, dextrose and lactose, used in a concentration of 1%, the maximum enzyme production was observed with maltose for both of the bacteria. The specific activity values of 17.85 and 24.85 U/g were found with the addition of maltose for ATCC 12980 and D413 amylases, respectively. These results are presented in Fig. 3.
As starch is an important substrate for fermentation experiments for amylases, and as it was found to be the second carbon choice for amylase production by isolate D413 (15,38 U/g) and strain ATCC 12980^T (13,6 U/g), the effects of different starch concentrations were also experimented as presented in Fig. 4. The optimum concentration for soluble starch was determined as 10 g/L and below this value a speed decline was observed especially in the case of ATCC 12980 amylase production.

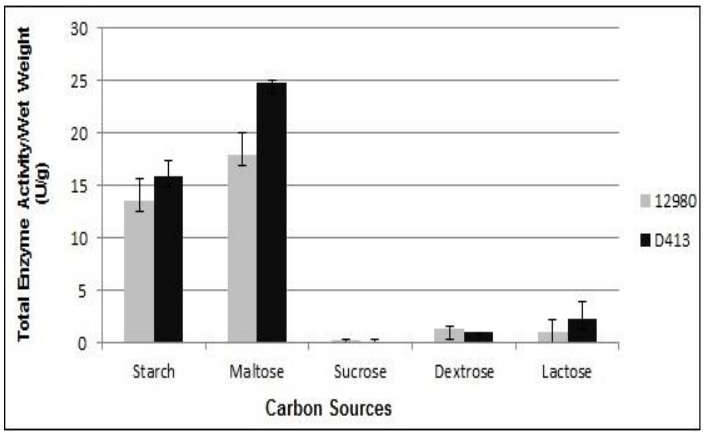

Figure 3 Effects of different carbon sources on amylase production by *G.stearothermophilus* ATCC 12980^T and *Geobacillus* sp. D413 at 55°C.

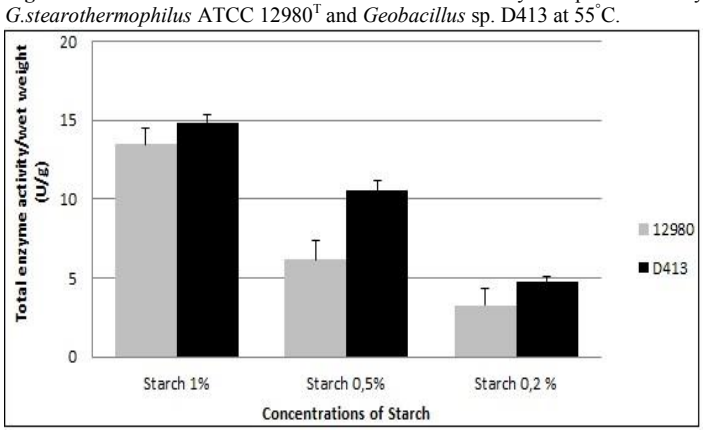

Figure 4 Effects of different concentrations of starch on amylase production by *G.stearothermophilus* ATCC 12980^T and *Geobacillus* sp. D413 at 55°C.

Effects of different nitrogen sources on amylase activities

When determining the effects of different nitrogen sources on amylase production, various concentrations of inorganic and organic compounds were added to the amylase production medium (Fig. 5). If only the inorganic nitrogen source ammonium chloride was used, the bacterial growth and enzyme production was inhibited. While the amylase production of strain D413 was diminished to 1.56 U/g, this value was found to be 0.59 U/g for strain ATCC 12980^T. In the other case, if the ammonium chloride in addition to tryptone or yeast extract were used with conjunction as double nitrogen sources, bacterial growth continued, but the enzyme production diminished for both of the bacteria ≤1 U/g). Therefore, it can be concluded that ammonium chloride had inhibitory effects for these α-amylase.

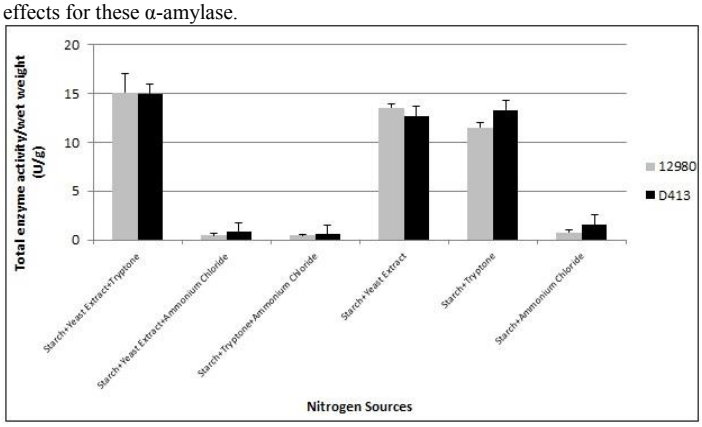

Figure 5 Effects of different nitrogen sources on amylase production by *G.stearothermophilus* ATCC 12980^T and *Geobacillus* sp. D413 at 55°C.

Among the organic nitrogen sources, neither_solely the yeast extract nor the tryptone addition increased the amylase activity for both of the enzymes. However, maximum enzyme production was observed at medium supplemented with both 0.5% yeast extract and 1% tryptone together as shown in Fig. 6. The amylase activities in the presence of yeast extract (0.5%) and tryptone (1%) were found to be 15.38 U/g and 13.6 U/g for D413 and ATCC 12980, respectively. If the concentrations of tryptone and yeast extract fell down in the medium, enzyme production of the reference strain showed a sharper decrease than the isolate D413. In a previous study of **Hamilton *et al.* (1999)** yeast extract turned out to be

the best nitrogen source for maximum amylase activity. However, **Saxena *et al.* (2007)** found the maximum amylase activity at medium supplemented with 0.5% peptone and 0.3% yeast extract for *Bacillus* sp. PN5. Therefore, it seems that the effects of various nitrogen sources also vary with the source organisms as in the case of carbon sources. As a consequence, the inorganic nitrogen source of 0.5 % ammonium chloride inhibit both the bacterial growth and amylase production; however, the organic sources like 0.5 % tryptone and 1 % yeast extract supported the growth and enzyme secretion for both strain ATCC 12980 and isolate D413. The effects of different carbon and nitrogen sources on the amylase production of these bacteria were also listed totally in Table 3.

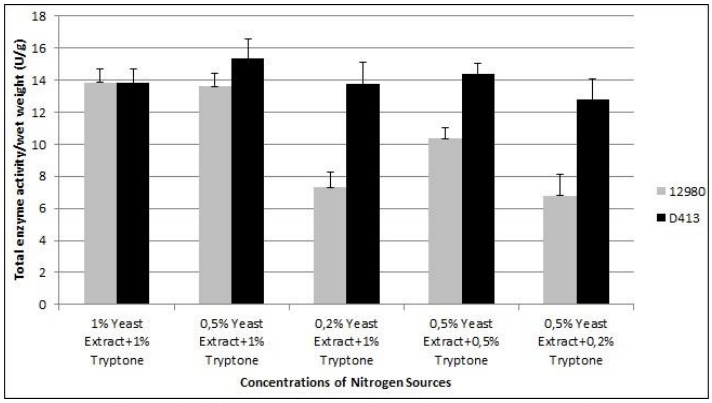

Figure 6 Effects of different concentrations of yeast extract and tryptone on amylase production by *G.stearothermophilus* ATCC 12980^T and *Geobacillus* sp. D413 at 55°C.

Table 3 Effects of different carbon and nitrogen sources on amylase production

Media (Content %)	*G. stearothermophilus* ATCC 12980		*Geobacillus sp*. D413	
	Enzyme activity (U/ml)	Enzyme activity/biomass (U/g)	Enzyme activity (U/ml)	Enzyme activity/biomass (U/g)
Starch + Yeast Extract + Tryptone (1; 0.5;1)	**0.95±0.16**	**13.6±1.48**	**1.23±0.05**	**15.38±1.59**
Starch + Yeast Extract + Tryptone (0.5; 0.5; 1.0)	0.55±0.10	6.19±1.21	0.73±0.04	10.53±0.66
Starch + Yeast Extract + Tryptone (0,2; 0.5;1.0)	0.16±0.04	3.29±1.03	0.38±0.02	4.79±0.29
Starch + Yeast Extract + Tryptone(1.0; 1.0; 1.0)	0.96±0.05	13.84±0.84	1.06±0.05	13.85±0.81
Starch + Yeast Extract + Tryptone (1.0; 0.2;1.0)	0.58±0.07	7.31±0.92	1.37 ±0.13	13.73±1.36
Starch + Yeast Extract + Tryptone (1.0; 0.5; 0.5)	0.73±0.05	10.36±0.64	1.44±0.21	14.38±2.10
Starch + Yeast Extract + Tryptone (1.0; 0.5; 0.2)	0.54±0.10	6.79±1.30	1.28±0.09	12.77±0.90
Starch + Yeast Extract (1.0; 0.5)	0.79±0.02	13.51±0.47	1.01±0.10	12.63±1.67
Starch + Tryptone (1.0;1.0)	0.92±0.04	11.48±0.54	1.06±0.10	13.32±1.35
Starch + Yeast Extract + NH_4Cl (1.0; 0.5; 0.5)	0.03±0.01	0.46±0.18	0.08±0.02	0.80±0.23
Starch + Tryptone + NH_4Cl (1.0; 1.0; 0.5)	0.03±0.008	0.44±0.07	0.06±0.01	0.55±0.10
Starch + NH_4Cl (1.0; 0.5)	0.02±0.001	0.59±0.21	0.05±0.01	1.56±0.33
Sucrose + Yeast Extract + Tryptone (1.0; 0.5;1.0)	0.01±0.008	0.21±0.018	0.01±0.007	0.14±0.11
Dextrose + Yeast Extract + Tryptone (1.0; 0.5; 1.0)	0.08±0.009	1.35±0.19	0.09±0.005	0.96±0.05
Lactose + Yeast Extract + Tryptone (1.0; 0.5; 1.0)	0.06±0.08	1.05±0.16	0.21±0.10	2.30±1.56
Maltose + Yeast Extract + Tryptone (1.0; 0.5; 1.0)	0.89±0.06	17.85±1.25	1.49±0.20	24.85±3.29

Effect of temperature and pH on enzyme activities and stabilities

Some distinctions were determined in both of these enzymes revealed by the temperature and pH activity and stabilities. The strain D413 extracellular α-amylase had temperature optima of 65°C and pH optima of 9.0. These values were found to be 65°C and pH 7.5 for strain ATCC 12980, respectively (Fig. 7a, b). After this temperature, especially for *Geobacillus* sp. D413, α-amylase activity was decreased. Both of these enzymes were very stable between temperature ranges from 45°C to 75°C and pH ranges from 4.0 to 10.5 (Fig. 8a, b). It is noteworthy that although the optimal growth pH (8.0) of ATCC 12980 was higher than D413 (pH 7.0) enzyme, the optimal amylase activity was observed in a value lower than D413 amylase. Notably, when the extracellular D413 α-amylase was incubated at 75°C for 10 min, it retained 65 % of its activity. This value was determined to be 54% for the reference strain. In addition, when D413 amylase was treated with buffers having pH:4.0 and 10.5 at 37°C for 15 h, it gained 98% and 86.5% of its activity, respectively. These were observed as pH 95% and 84.5% for ATCC 12980^T amylase.

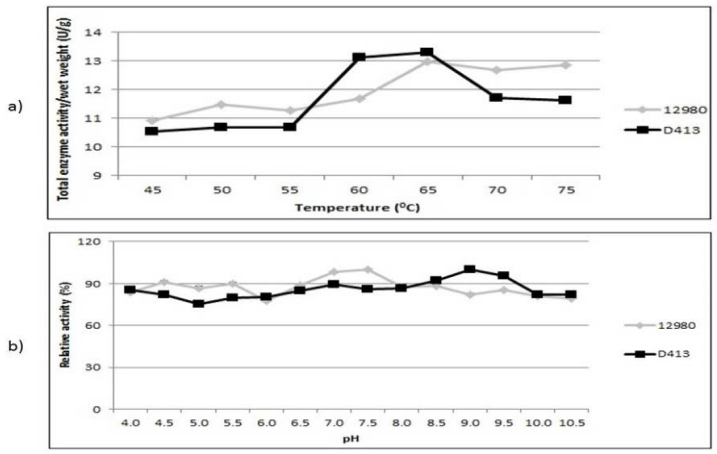

Figure 7 Effects of *G.stearothermophilus* ATCC 12980^T (◊) and *Geobacillus* sp. D413 (□) amylase activities under different a) temperatures at pH 7.0 and b) pH values at 65°C.

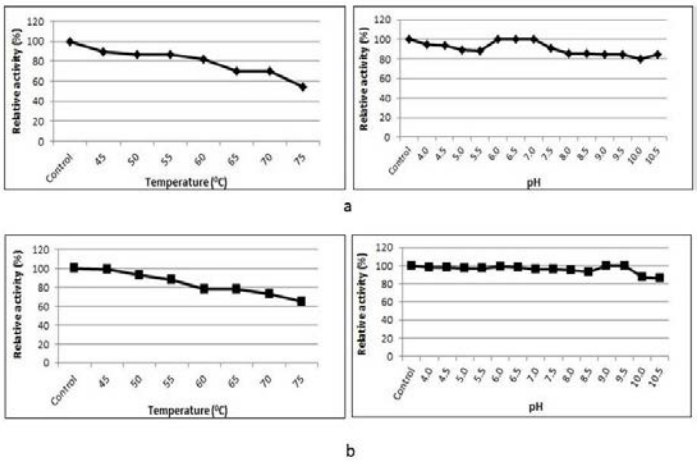

Figure 8 Temprerature and pH stabilities of amylases from a) *G. stearothermophilus* ATCC 12980^T (◊) and b) *Geobacillus* sp. D413 (□)

DISCUSSION

Amylases are among the most important enzymes and are of great significance in biotechnological processes. Particularly thermostable alpha amylases are used in many industrial processes at high temperature. In the present paper, we report the characterization of novel thermostable amylase produced by thermophilic strain *Geobacillus* sp. D413, which efficiently hydrolyzes the starch biomass. As a result of scanning performed amylase activity; *Geobacillus* sp. D413 has been selected as the best enzyme producing isolate. Enzyme production of *Geobacillus* sp. D413 has been optimized and used in *G. stearothermophilus* ATCC 12980.

Culture conditions (medium pH, incubation temperature, incubation time, and carbon and nitrogen sources) were determined for maximum enzyme production. For the maximum enzyme production optimal temperature and pH was found to be 55°C and pH:7.0, respectively for our isolate. In general, most of the thermophilic members from *Bacillaceae* family were reported to produce maximum enzyme production at temperatures ranging from 35°C to 60°C **(Kiran *et al.*, 2005; Dheeran *et al.*, 2010; Suman and Ramesh, 2010).** On the other hand, the enzyme production was found to be inhibited (≤1.0 U/g) when sucrose, lactose and glucose were used as carbon sources. The previous studies have revealed that especially production of carbohydrate degrading enzymes in most species of the genus *Bacillus* is exposed to catabolic repression being readily metabolized in the presence of substrates such as glucose **(Lin *et al.*, 1998).** As starch is an important substrate for fermentation experiments for amylases and as it was found to be the second carbon choice for amylase production by isolate D413 (15.38 U/g) and strain ATCC 12980^T (13.6 U/g). Also, the enzyme activity was found to be associated with the starch concentration. Therefore, a direct proportion was observed between the starch concentration in the medium and the amylase production for both of the bacteria. Similar to this finding, **Suman and Ramesh (2010)** demonstrated the optimum concentration of soluble starch to be 20 g /L for *Bacillus* strain KCPSS-12ss amylase.

The optimal temperature and pH for α-amylase activities were within the ranges (30 to 105°C) of the reported α-amylases from endospore-forming bacilli **(Teodoro and Martins, 2000; Ezeji and Bahl, 2006; Asgher *et al.*, 2007; Saxena *et al.*, 2007; Arikan, 2008; Asoodeh *et al.*, 2010).** Among the previous studies, **Ezeji and Bahl (2006)** reported that *G. thermodenitrificans* HRO10 amylase retained 53.4% of its original activity after incubation without substrate at 70°C for 10 min. These results are in agreement with the reports of **Ezeji and Bahl (2006)** and **Teodoro and Martins (2000)**; and in those studies optimum temperature was diminished beyond of 55°C and was inactivated at 95°C for ten min. Also **Oziengbe and Onilude (2012)** have determined that deterioration took place in the stability of *B. licheniformis* amylase after temperature above 70°C.

Similar results were reported in the study of **Asgher *et al.*, 2007**. In that study, they found that the a-amylase of *B. subtilis* JS-2004 strain lost only its %6 of its original activity after incubation for 24 h at its optimum pH. But in the study of **Ezeji and Bahl (2006)**, *G. thermodenitrificans* HRO10 α-amylase showed maximum activity in an acidic pH such as 5.5. Therefore, when compared with the ATCC 1280 and other source microorganisms, the extracellular α-amylase of D413 differed from other bacilli enzymes by means of its temperature and pH optima values, by its high thermostability at its optimal temperature and also by its broad pH stabilities. Especially the α-amylase of D413 can be more active and stable on an alkaline pH such as 9.0, and differed from ATCC 12980 enzymes having optimum pH 7.0 at neutral conditions.

CONCLUSION

Microbial α-amylases are in use in industrial processes occupying approximately 30% of the world enzyme market. As in these processes the desired parameters are thermal and pH stability, the α-amylase production, activity and stability conditions were determined for optimal amylase production from *Geobacillus* sp. D413 by comparing the reference strain *G. stearothermophilus* ATCC 12980 in this study. As a result of the optimization studies, it was observed that when D413 enzyme was incubated at 55°C and pH 7.0 in media containing 1% soluble starch, 0.5% yeast extract and 1% tryptone, it showed maximum activity at pH 9.0 and at 65°C. D413 α-amylase enzyme was also found to be stable at 75°C and pH 10.5. *Geobacillus* sp. D413 α-amylase possesses high conformational stability at elevated temperatures, has an optimal pH at alkaline environments and shows activity in broad pH ranges; all these characteristic properties of the extracellular *Geobacillus* sp. D413 α-amylase are required parameters in enzymes desired for biotechnologically important industrial applications. Thus, when its pH optima and conformational stability are considered, the D413 α-amylase may prove to have a potential in biotechnological processes such as hydrolysis of starch to glucose.

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EFFECTIVE IDENTIFICATION OF *LACTOBACILLUS PARACASEI* SSP. *PARACASEI-1* BY 16S-23S rRNA INTERGENIC SPECER REGION SEQUENCING

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ABSTRACT

Fermentation profiling is a common tool to identify *Lactobacillus paracasei* from other related members of *Lactobacillus* genus. Because of the proximate biochemical characteristics, identification based on the fermentation pattern within *L. casei* group (*L. casei, L. paracasei* and *L. rahmnosus*) has considerable limitation. On the other hand, members of *L. casei* group are genetically very closely related and sequencing of signature region is the most reliable and rapid method of differentiation. In this study, instead of sequencing entire 16S or 23S ribosomal RNA gene, we have sequenced 16s-23s rRNA intergenic spacer region and compared them across the members of *L. casei* group. We also compiled thirteen signature sequences within 16s-23s ISR rRNA gene of *L. casei* group members. Our isolated strain (*L. paracasei* ssp. *paracasei-1*) resembled 100% and 99% similarity when compared with 16s-23s rRNA intergenic spacer region of *L. paracasei* and *L. casei* respectively. Our study summarizes that sequencing of short 16s-23s rRNA intergenic spacer region carries great significance in identification of closely related probiotic bacteria such as the members of *L. casei* group. The findings of this research could be very much helpful for food and pharmaceutical industries who are dealing with probiotic bacteria.

Keywords: Identification, *L. paracasei* ssp. *paracasei-1*, 16s-23s rRNA, signature sequence, probiotic

INTRODUCTION

The genus *Lactobacillus* is subject to continual taxonomic changes. It is often thorny to use classical microbiological methods to identify lactic acid bacteria even to genus level because of their similar nutritional and growth requirements (**Dubernet *et al.*, 2002**). Moreover, identifying a *Lactobacillus* isolate accurately to the species level requires about 17 phenotypic tests (**Tannock *et al.*, 1999**). Even bigger challenge is to accurately identify the species of *L. casei* group based on phenotypic tests (**Ward and Timmins, 1999**). However, confirming the identification of diverse species of lactic acid bacteria is very important for quality control and quality assurance perspective when it is intended for human use (**Tilsala *et al.*, 1997**). Very often identification of *Lactobacillus* spp. appears as a dilemma to the researchers. *L. casei* group is an ideal example which must overcome some unusual and difficult obstacles in order to pacify confusion in their identification, especially within the *L. casei* group members. The taxonomy of *L. casei* group has been in dispute over the transfer of some species and strains from *L. casei* to *L. paracasei* (**Kisworo *et al.*, 2008**). *L. casei* group gone through several rearrangement notably from five member group to four members enlisted in *Bergy's Manual* of *Systematic Bacteriology* (*L. casei* ssp. *alactosus* was combined with *L. casei* ssp. *casei*) (**Mori *et al.*, 1997**). In 1989 **Collins *et al.* (1989)** reclassified the subspecies of *L. casei* into *L. casei, Lactobacillus paracasei,* and *Lactobacillus rhamnosus* on the basis of DNA-DNA relatedness data. Although **Coudeyras *et al.* (2008)** classified *L. casei* into four group members, however, we have considered *L. casei* with three group members including *L. casei*, *L. paracasei* and *L. rhamnosus* based on the latest phylogenic structure revealed by **Salvetti *et al.* (2012).**

Considering these identification enigma, use of a diagnostic marker specific to the probiotic strain of interest carries significant importance. Moreover, the beneficial characteristics associated with the probiotic are strain specific (**Flint *et al.*, 2005).** Therefore nucleotide base sequences of *Lactobacillus* 16S ribosomal DNA (rDNA) is an important reliable analysis which provides an accurate basis for identification (**Tannock *et al.*, 1999**). **Barry *et al.* (1991)** have shown that the ribosomal intergenic regions are more variable between bacterial species compared to 16S and 23S rRNA genes. **Tannock *et al.* (1999)** documented several studies which have demonstrated that the DNA sequence between the 16S and 23S genes of lactobacilli is hypervariable. This intergenic spacer region (ISR) is about 200 bases in length. The 16s-23s spacer sequences of lactobacilli are adequately species specific which can be used as a marker to identify *Lactobacillus* species. In fact, using primer that will amplify 16s-23s ISR region is a relatively simple and quick method through which lactobacilli can be identified without resorting to the use of species-specific PCR primers (**Tannock *et al.*, 1999**). While drawing significant relationship within closely related bacterial species, sequencing comparisons are often applied. **Mori *et al.* (1997)** compared the 16S rRNA sequences of all strains of the *L. casei* subspecies. **Chen *et al.* (2000)** reported intergroup comparisons of the 23S-5S rRNA spacer genes from *Lactobacillus casei* related strains. In this study we have compared the sequences of 16s-23s ISR region of *L. casei* group along with the identification of *L. paracasei* ssp. *paracasei-1*.

Hoque *et al.* (2010) reported the genus of a bacterial isolates as *Lactobacillus* spp. in their study. Based on classical microbiology and biochemical reaction, **Islam *et al.* (2012)** identified the same isolated bacteria reported by **Hoque *et al.* (2010)** as *L. paracasei* ssp. *paracasei-1*. Following that, **Honi *et al.* (2013)** also bolstered the identification, reported by **Islam *et al.* (2012),** as *L. paracasei* ssp. *paracasei-1* on the basis of PCR amplification of 16s-23s ISR region**.** However, none of these reports include sequencing of the ISR region of *L. paracasei* ssp. *paracasei-1*. *L. paracasei* and other members of *L. casei* group share extremely high similarities within the 16s-23s ISR region**.** So there is every possibility of getting a false positive result from PCR amplification based identification while identifying *L. paracasei* without the sequencing of 16s-23s ISR region. Sequencing of the 16s-23s ISR region of previously identified *L. paracasei* ssp. *paracasei-1* (**Islam *et al.*, 2012; Honi *et al.*, 2013**) and comparing the sequenced results across the already reported 16s-23s ISR region sequences of *L. casei* group members, in order to determine signature sequences, are the main focus of this research.

MATERIAL AND METHODS

Collection of test organism

The test organism (*L. paracasei* ssp. *paracasei-1*, isolated from regional yogurt) (**Islam *et al*., 2012; Honi *et al*. 2013**) was collected from the stock culture maintained by the Biochemistry and Molecular biology Laboratory of Biotechnology & Genetic Engineering Discipline, Khulna University.

Subculture of the bacteria

The test organism was grown in MRS broth, added with 0.05% cysteine, (HiMedia/India) and MRS agar (HiMedia/India) plated in an incubator (Shellab/USA) at 37^0C for 16 hrs. pH (Mettler Toledo/Switzerland) of the media was adjusted to 6.5 (**Islam *et al*., 2012; Honi *et al*., 2013**).

Morphological, physiological and biochemical identification

Identification of *Lactobacillus* spp. was verified via gram staining, cell morphology, endospore assay, catalase test, motility test, colony morphology (**Hoque *et al*., 2010; Islam *et al*., 2012; Honi *et al*., 2013**).

Extraction of chromosomal DNA

Chromosomal DNA was extracted from overnight MRS culture, stored at 37^0C, using bacterial DNA preparation kit (jena bioscience/Germany) according to the manufacturer's instructions. Quantity of DNA extracts was checked with a spectrophotometer (Shimadzu: UV-1800 /Japan) (**Skelin *et al*., 2012**).

Quantification of genomic DNA of *Lactobacillus* spp.

Isolation of genomic DNA was confirmed by using horizontal gel electrophoresis system (BioRad: MINI SUB CELL /USA) and gel documentation system (CB Scientfic: ALPHAIMAGER HP /USA). In this case 2% agarose was used. Then quantification of DNA was done by using spectrophotometer. Absorption reading was taken at 260, 280 and 320 nm. Purity, concentration and total yield of DNA were calculated from these readings which are detailed in Table 1.

Table 1 Concentration and purity of extracted DNA

Sample	A260	A280	A320	Concentration[1], µg/ml	Purity[2]	Purity[3]	DNA Yield[4], µg
A	0.412	0.24	0.008	505	1.71	1.74	1010
B	0.424	0.24	0.012	515	1.76	1.80	1030
C	0.448	0.28	0.02	535	1.60	1.64	1070

1 = [(A260-A320) × Dilution Factor (= 25) × 50 µg/ml pure ds DNA]
2 = (A260÷A280)
3 = [(A260-A320)÷(A280-A320)]
4 = [(DNA Concentration in µg/ml × Total Sample volume (=2 ml)]
A260 = Absorption at 260 nm, A280 = Absorption at 280 nm, A320 = Absorption at 320 nm

PCR amplification

To determine the identification up to species level of *L. paracasei* isolates, species-specific PCR assay was carried out with *Lactobacillus paracasei* species-specific primer set, supplied by Bioneer Corp., USA. Primer set Lp1F- (5'-GGGGATCACCCTCAAGCACCCT -3') and Lp1R-(5'-GCGTCAGCGGTTATGCGATGC -3') along with Applied Biosystem's thermal cycler (VERITY/USA) were used **(Honi *et al*., 2013)**. Amplification of DNA from the 16s-23s ISR rRNA gene was carried out using promega PCR kit (Promega Corporation/USA) and PCR operating protocol described by **Honi *et al.* (2013). Table 2** gives information on primer details and **Table 3** explains the PCR sample loading detail. Amplification products were separated on a 2% agarose gel using horizontal gel Electrophoresis System (BioRad: MINI SUB CELL /USA), stained with 2.5% ethidium bromide, visualized and documented using a Gel Documentation System (CB Scientfic: ALPHAIMAGER HP /USA) **(Honi *et al*., 2013).** The amplified PCR products were purified using conventional ethanol based purification method and manufacturer's instruction of BigDye XTerminator Purification kit (AB Science, USA).

Table 2 Primers used for PCR amplification of 16s-23s intergenic spacer region

Primer	Sequences (5' > 3')	Length	Start	Stop	Tm	GC%
Lp1F	GGGGATCACCCTCAAGCACCCT	22	63	84	64.0	63.64
Lp1R	GCGTCAGCGGTTATGCGATGC	21	208	188	64.3	61.90

Table 3 PCR reaction mixture

Sl No.	Reagents	PCR Master Mix (µl)	Forward Primer[1] (µl)	Reverse Primer[1] (µl)	DNA Template[2] (µl)	DNA Template[3] (µl)	Nuclease Free Water (µl)	Total Vol. (µl)
	Sample							
1	Sample 1	12.5	0.5	0.5	3	0	8.5	25
2	Sample 2	12.5	0.5	0.5	3	0	8.5	25
3	Sample 3	12.5	0.5	0.5	3	0	8.5	25
4	Sample 4	12.5	0.5	0.5	0	3	8.5	25
5	Sample 5	12.5	0.5	0.5	0	3	8.5	25
6	Sample 6	12.5	0.5	0.5	3	0	8.5	25
7	Sample 7	12.5	0.5	0.5	3	0	8.5	25
8	Sample 8	12.5	0.5	0.5	3	0	8.5	25
9	Sample 9	12.5	0.5	0.5	0	3	8.5	25
10	Sample10	12.5	0.5	0.5	0	3	8.5	25

1= Stock Concentration (100 nano mole)
2= Sample-B of Table 1
3= Sample-C of Table 1

16s-23s ISR DNA sequence

The PCR products of the amplification were used for re-identification at the species level and to recheck the primer specificity for *Lactobacillus paracasei* ssp. *paracasei-1* as reported by **Honi *et al.* (2013)**. For DNA sequencing we have used sequencing kit (AB science, USA). An in-house developed protocol was used in determining the sequencing. Briefly, in each cycle, during initial denaturation the temperature was increased to 96°C for 1 minute, followed by 96°C and 10 seconds denaturation cycle. Then temperature decreased to 50°C for 5 seconds to anneal the primer to the template, and finally increased to 60°C for 4 minutes during elongation step involving enzymatic synthesis. A total of 25 cycles were completed. Finally the reaction was kept at 4°C. The PCR product was purified using ethanol precipitation method and purification kit. **Table 4** contains the cycle sequencing sample loading detail which was performed in a DNA analyzer unit of Applied Biosystem science (Applied Biosystem: 3500 GENETIC ANALYZER /USA). The obtained sequences were aligned and compared with the *L. casei* group member's 16s-23s rRNA ISR sequences (accession number U32964.1, U32966.1 and AF121200.1) stored at GenBank database (National Center for Biotechnology Information, Bethesda, MD, USA). BLAST and ClustalW2 algorithm were used in this comparison. To test the reliability of the cycle sequencing method, we have sequenced pGEM vector (AB Science, USA) using the same sequencing protocol described above.

Table 4 Cycle sequencing sample preparation:

Sl No.	Reagents	Ready Reaction Mix (µl)	Dilution Buffer (µl)	DNA Template[1], µl	Forward Primer[2], µl	Reverse Primer[2], µl	Nuclease Free Water (µl)	Total Vol. (µl)
	Sample							
1	Sample 1	1.0	1.5	1.5[a]	2.0[b]	0	4.0	10
2	Sample 2	1.0	1.5	1.5	2.0	0	4.0	10
3	Sample 3	1.0	1.5	1.5	0	2.0	4.0	10

1= Sample B of Table 1, 2= Concentration is 100 nano mole, a= pGEM control, b= Forward primer for pGEM

Comparison of 16s-23s ISR of *L. casei* group

Tilsala *et al.* (1997) determined the 16s-23s rRNA sequence of *L. paracasei* (accession number U32964.1) and *L. Rhamnosus* (accession number U32966.1). **Tannock *et al.* (1999)** determined the 16s-23s rRNA sequence of *L. casei* ATCC 334 (accession number AF121200.1). We tried to identify the signature sequences of ISR region within the *L. casei* group. The homology between *L. paracasei* ssp. *paracasei-1* and *L. casei* group members were determined in order to differentiate *L. paracasei* ssp. *paracasei-1* from *L. casei* group members based on 16s-23s ISR rRNA sequencing. Finally we compared 16s-23s ISR rRNA gene sequence of *L. paracasei* ssp. *paracasei-1* with the 16s-23s ISR rRNA regions of *L. casei* and *L. paracasei* to confirm the identity based on sequencing similarity pattern. All the FASTA sequences were obtained from National Center for Biotechnology Information (NCBI).

Three types of alignment were performed using Clustal W2:

1. Alignment of 16s-23s ISR sequence of *L. casei* group (*L. paracasei,* GenBank U32964.1, *L. rhamnosus* (U 32966.1) and *L. casei*, (AF121200.1))
2. Alignment of 16s-23s *L. paracasei* (U32964.1) and *L. casei* (AF121200.1)
3. Alignment of 16s-23s *L. paracasei* (U32964.1), *L. casei* (AF121200.1) and *L. paracasei* ssp. *paracasei-1*

RESULTS

Morphological, physiological and biochemical characterization

The isolate showed their colonies morphology small, circular, white-creamy in colors. Microscopically they were gram-positive rod shaped, non-motile, catalase negative and non spore forming. Their colony and microbiological characteristics have been summarized in **Table 5**. According to their characteristics, the isolates were identified as *Lactobacillus* spp.

Table 5 Different characteristics of isolated *Lactobacillus* spp.

Morphological, physiological and biochemical test	Findings
Gram staining	Positive
Cell morphology	Rod shaped
Endospore staining	Negative
Catalase test	Negative
Motility test	Negative
Colony morphology	Small round, creamy white

Extraction and quantification of genomic DNA from *Lactobacillus* spp.

After performing gel electrophoresis (2% agarose, voltage: 85V) there was a distinctive band of genomic DNA (Figure 1). Quantification of DNA concentration carried out by spectrophotometer. The purity range of isolated DNA of the test organism was within 1.60 to 1.80 **(Table 1)**

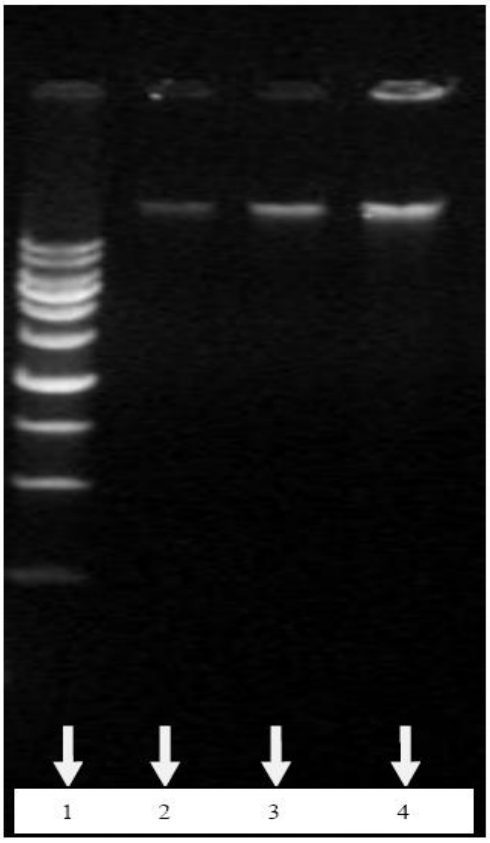

Figure 1 Isolated genomic DNA of *Lactobacillus* spp. (1= 1Kb ladder, 2= sample A, 3= sample B and 4= sample C of Table 1

PCR amplification of the 16s-23s rRNA ISR from *Lactobacillus paracasei* DNA

One primer pair (Lp1F & Lp1R) was used for PCR amplification of spacer region of our *Lactobacillus* spp. PCR product was obtained with the primer pair (Figure 2). The amplified product with the same primer pair has been reported to be 146 bp long (**Honi *et al.*, 2013**).

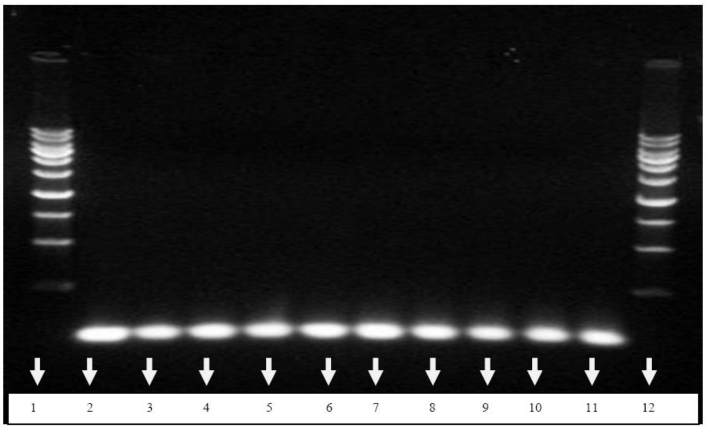

Figure 2 Gel run of PCR amplified sample

Lane 1: 1Kb Ladder	Lane 2: Sample-1	Lane 3: Sample-2	Lane 4: Sample-3	Lane 5: Sample-4	Lane 6: Sample-5
Lane 7: Sample-6	Lane 8: Sample-7	Lane 9: Sample-8	Lane 10: Sample-9	Lane 11: Sample-10	Lane 12: 1Kb Ladder

16s-23s ISR rRNA gene sequencing result

Obtained forward sequence using forward primer Lp1F
>
TTGAAAACTGGATATCATTGTATTAATTGTTTTAAATTGCCGAGAACACAGCGTATTTGTATGAGTTTCTGAA
AAAGAAATTCGCATCGCATAACCGCTGACGCAA

Query sequence (obtained forward sequence) **:**

```
1  TTGAAAACTG GATATCATTG TATTAATTGT TTTAAATTGC CGAGAACACA GCGTATTTGT
61 ATGAGTTTCT GAAAAAGAAA TTCGCATCGC ATAACCGCTG ACGCAA
```

Subject sequence (*Lactobacillus paracasei paracasei*, 16s-23s rRNA spacer region, U32964.1)

```
1   CTAAGGAAAC AGACTGAAAG TCTGACGGAA ACCTGCACAC ACGAAACTTT GTTTAGTTTT
61  GAGGGGATCA CCCTCAAGCA CCCTARCGGG TGCGACTTTG TTCTTTGAAA ACTGGATATC
121 ATTGTATTAA TTGTTTTAAA TTGCCGAGAA CACAGCGTAT TTGTATGAGT TTCTGAAAAA
181 GAAATTCGCA TCGCATAACC GCTGACGCAA GTCAGTACAG G
```

```
Sequence ID: lcl|49579Length: 221Number of Matches: 1

Score              Expect      Identities        Gaps

196 bits(106)      1e-55       106/106(100%)     0/106(0%)

Query  1    TTGAAAACTGGATATCATTGTATTAATTGTTTTAAATTGCCGAGAACACAGCGTATTTGT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  105  TTGAAAACTGGATATCATTGTATTAATTGTTTTAAATTGCCGAGAACACAGCGTATTTGT  164

Query  61   ATGAGTTTCTGAAAAAGAAATTCGCATCGCATAACCGCTGACGCAA  106
            ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  165  ATGAGTTTCTGAAAAAGAAATTCGCATCGCATAACCGCTGACGCAA  210
```

Among 221 bases of reference sequence 106 bases perfectly matched with the results of sample's forward sequence.

Obtained reverse sequence using reverse primer Lp1 R:
>
ATACAAATACGCTGTGTTCTCGGCAATTTAAAACAATTAATACAATGATATCCAGTTTTCAAAGAACAAAGT
CGCACCCGYTAGG

Blasting was performed in following way

Query sequence (obtained reverse sequence)**:**

```
1  ATACAAATAC GCTGTGTTCT CGGCAATTTA AAACAATTAA TACAATGATA TCCAGTTTTC
61 AAAGAACAAA GTCGCACCCG YTAGG
```

Subject sequence (*Lactobacillus paracasei paracasei*, 16s-23s rRNA Spacer Region, U32964.1):

```
1   CTAAGGAAAC AGACTGAAAG TCTGACGGAA ACCTGCACAC ACGAAACTTT GTTTAGTTTT
61  GAGGGGATCA CCCTCAAGCA CCCTARCGGG TGCGACTTTG TTCTTTGAAA ACTGGATATC
121 ATTGTATTAA TTGTTTTAAA TTGCCGAGAA CACAGCGTAT TTGTATGAGT TTCTGAAAAA
181 GAAATTCGCA TCGCATAACC GCTGACGCAA GTCAGTACAG G
```

```
Sequence ID: lcl|19777Length: 221Number of Matches: 1

Score              Expect      Identities        Gaps

154 bits(83)       6e-43       85/85(100%)       0/85(0%)

Query  1    ATACAAATACGCTGTGTTCTCGGCAATTTAAAACAATTAATACAATGATATCCAGTTTTC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  166  ATACAAATACGCTGTGTTCTCGGCAATTTAAAACAATTAATACAATGATATCCAGTTTTC  107

Query  61   AAAGAACAAAGTCGCACCCGYTAGG  85
            |||||||||||||||||||||||||
Sbjct  106  AAAGAACAAAGTCGCACCCGYTAGG  82
```

Among 221 bases of reference sequence 85 bases perfectly matched, shown in bold, with the results of sample's reverse sequence. Sequence given below, shown within box, shows the forward primer position within the reference sequence. Good signal sequence was obtained after 20 initial bases which continued up to 210 no. base.

Forward

```
1   CTAAGGAAAC AGACTGAAAG TCTGACGGAA ACCTGCACAC ACGAAACTTT GTTTAGTTTT
61  GAGGGGATCA CCCTCAAGCA CCCTARCGGG TGCGACTTTG TTCTTTGAAA ACTGGATATC
121 ATTGTATTAA TTGTTTTAAA TTGCCGAGAA CACAGCGTAT TTGTATGAGT TTCTGAAAAA
181 GAAATTCGCA TCGCATAACC GCTGACGCAA GTCAGTACAG G
```

The following sequence, shown within box, shows the reverse primer position within the reference sequence. Good signal sequence was obtained after 21 bases which continued up to 82 no. base.

Reverse

```
1   CTAAGGAAAC AGACTGAAAG TCTGACGGAA ACCTGCACAC ACGAAACTTT GTTTAGTTTT
61  GAGGGGATCA CCCTCAAGCA CCCTARCGGG TGCGACTTTG TTCTTTGAAA ACTGGATATC
121 ATTGTATTAA TTGTTTTAAA TTGCCGAGAA CACAGCGTAT TTGTATGAGT TTCTGAAAAA
181 GAAATTCGCA TCGCATAACC GCTGACGCAA GTCAGTACAG G
```

After combining both the results it was found that almost 148 bases, shown within box, were completely matched with the reference sequence.

Combined result:

```
1   CTAAGGAAAC AGACTGAAAG TCTGACGGAA ACCTGCACAC ACGAAACTTT GTTTAGTTTT
61  GAGGGGATCA CCCTCAAGCA CCCTARCGGG TGCGACTTTG TTCTTTGAAA ACTGGATATC
121 ATTGTATTAA TTGTTTTAAA TTGCCGAGAA CACAGCGTAT TTGTATGAGT TTCTGAAAAA
181 GAAATTCGCA TCGCATAACC GCTGACGCAA GTCAGTACAG
```

Sequences of the 16s-23s ISR rRNA gene of isolated *L. paracasei* ssp. *paracasei-1*

From the combined cycle sequencing result we did determine the intended region designed for our primer. Sequence of *L. paracasei* ssp. *paracasei-1* showed 100% similarity (comparison - 3) with the 16s-23s ISR sequence of *L. paracasei* (accession number U32964.1).

Comparison of 16s-23s ISR rRNA gene of *L. casei* group and *L. paracasei* ssp. *paracasei-1*

The 16s-23s ISR rRNA gene sequences of the *L. casei* group species obtained from the data bank (accession number U32964.1, U32966.1 & AF121200.1) and shown in comparison 1. While comparing *L. casei* and *L. paracasei* sequences we saw that *L. paracasei* possess four extra bases at position no 1, 2, 220 & 221 (comparison 2). Apart from that, we observed a single base difference at 86 no. position. Then we blasted ISR sequence of *L. paracasei* ssp. *paracasei-1* with *L. paracasei* and *L. casei* in order to confirm the identification. From these blasts (comparisons 1 – 3, Table 6 & 7) we became 100% ascertain that our isolate was *L. paracsei* ssp. *paracasei-1.*

Table 6 Signature sequences between the members of *L. casei group* and *L. paracasei* ssp. *paracasei-1*

Nucleotide position[1]	**1**	**2**	**69**	**86**	**126**	**127**	**131**	**175**	**177**	**179**	**180**	**221**	**222**
*L. rhamnosus (*U32966.1)	*C*	*T*	*T*	*G*	*T*	*G*	*A*	*A*	*T*	*A*	*T*	*G*	*G*
L. paracasei (U32964.1)	*C*	*T*	*C*	***R***	*A*	*T*	*T*	*G*	*A*	-	*A*	*G*	*G*
L. casei (AF121200.1)	-	-	*C*	***A***	*A*	*T*	*T*	*G*	*A*	-	*A*	-	-
L. paracasei ssp. *paracasei-1*			*C*	***R***	*A*	*T*	*T*	*G*	*A*	-	*A*		

1= From the 5' end of the 16s-23s ISR rRNA sequence of *L. rhamnosus*

Table 7 Comparison of 16s-23s ISR rRNA region of isolated *L. paracasei* ssp. *paracasei-1* with *L. casei* group members

Strain	Homology with the sequence of *L. paracasei*- ssp. *paracasei-1*	Sequencing start-end position[1]	Position no. where difference were found [1]
L. paracasei (U32964.1)	100%	63-210	None
L. casei (AF121200.1)	99%	63-210	86
L. rhamnosus (U32966.1)	93%	63-210	69, 86, 126, 127, 131, 175, 177, 178,179,180,

1= Numbering position with respect to *L. rhamnosus*

Comparison 1 CLUSTAL 2.1 multiple sequence alignment no. 01 (*L. rhamnosus* (U32966.1), *L. paracasei* (U32964.1) and *L. casei* (AF121200.1))

```
gi|1854588|gb|U32966.1|LRU3296      CTAAGGAAACAGACTGAAAGTCTGACGGAAACCTGCACACACGAAACTTT 50
gi|1854587|gb|U32964.1|LPU3296      CTAAGGAAACAGACTGAAAGTCTGACGGAAACCTGCACACACGAAACTTT 50
gi|5902977|gb|AF121200.1|           --AAGGAAACAGACTGAAAGTCTGACGGAAACCTGCACACACGAAACTTT 48
                                      **************************************************

gi|1854588|gb|U32966.1|LRU3296      GTTTAGTTTTGAGGGGATTACCCTCAAGCACCCTAGCGGGTGCGACTTTG 100
gi|1854587|gb|U32964.1|LPU3296      GTTTAGTTTTGAGGGGATCACCCTCAAGCACCCTARCGGGTGCGACTTTG 100
gi|5902977|gb|AF121200.1|           GTTTAGTTTTGAGGGGATCACCCTCAAGCACCCTAACGGGTGCGACTTTG 98
                                    ****************** ***************** **************

gi|1854588|gb|U32966.1|LRU3296      TTCTTTGAAAACTGGATATCATTGTTGTAAATGTTTTAAATTGCCGAGAA 150
gi|1854587|gb|U32964.1|LPU3296      TTCTTTGAAAACTGGATATCATTGTATTAATTGTTTTAAATTGCCGAGAA 150
gi|5902977|gb|AF121200.1|           TTCTTTGAAAACTGGATATCATTGTATTAATTGTTTTAAATTGCCGAGAA 148
                                    *************************: ***:******************

gi|1854588|gb|U32966.1|LRU3296      CACAGCGTATTTGTATGAGTTTCTAATAATAGAAATTCGCATCGCATAAC 200
gi|1854587|gb|U32964.1|LPU3296      CACAGCGTATTTGTATGAGTTTCTGAAA-AAGAAATTCGCATCGCATAAC 199
gi|5902977|gb|AF121200.1|           CACAGCGTATTTGTATGAGTTTCTGAAA-AAGAAATTCGCATCGCATAAC 197
                                    ************************.*:* :********************

gi|1854588|gb|U32966.1|LRU3296      CGCTGACGCAAGTCAGTACAGG 222
gi|1854587|gb|U32964.1|LPU3296      CGCTGACGCAAGTCAGTACAGG 221
gi|5902977|gb|AF121200.1|           CGCTGACGCAAGTCAGTACA-- 217
                                    **********************
```

Comparison 2 CLUSTAL 2.1 multiple sequence alignment no. 03 (*L. paracasei* (U32964.1) and *L. casei* (AF121200.1))

```
gi|1854587|gb|U32964.1|LPU3296      CTAAGGAAACAGACTGAAAGTCTGACGGAAACCTGCACACACGAAACTTT 50
gi|5902977|gb|AF121200.1|           --AAGGAAACAGACTGAAAGTCTGACGGAAACCTGCACACACGAAACTTT 48
                                      **************************************************

gi|1854587|gb|U32964.1|LPU3296      GTTTAGTTTTGAGGGGATCACCCTCAAGCACCCTARCGGGTGCGACTTTG 100
gi|5902977|gb|AF121200.1|           GTTTAGTTTTGAGGGGATCACCCTCAAGCACCCTAACGGGTGCGACTTTG 98
                                    ********************************** **************

gi|1854587|gb|U32964.1|LPU3296      TTCTTTGAAAACTGGATATCATTGTATTAATTGTTTTAAATTGCCGAGAA 150
gi|5902977|gb|AF121200.1|           TTCTTTGAAAACTGGATATCATTGTATTAATTGTTTTAAATTGCCGAGAA 148
                                    **************************************************

gi|1854587|gb|U32964.1|LPU3296      CACAGCGTATTTGTATGAGTTTCTGAAAAAGAAATTCGCATCGCATAACC 200
gi|5902977|gb|AF121200.1|           CACAGCGTATTTGTATGAGTTTCTGAAAAAGAAATTCGCATCGCATAACC 198
                                    **************************************************

gi|1854587|gb|U32964.1|LPU3296      GCTGACGCAAGTCAGTACAGG 221
gi|5902977|gb|AF121200.1|           GCTGACGCAAGTCAGTACA-- 217
                                    **********************
```

Comparison 3 CLUSTAL 2.1 multiple sequence alignment (*L. paracasei* (U32964.1), *L. casei* (AF121200.1) and *L. paracasei* ssp. *paracasei-1* (Our isolate))

```
gi|1854587|gb|U32964.1|LPU3296      CTAAGGAAACAGACTGAAAGTCTGACGGAAACCTGCACACACGAAACTTT 50
gi|5902977|gb|AF121200.1|           --AAGGAAACAGACTGAAAGTCTGACGGAAACCTGCACACACGAAACTTT 48
gi|KU|gb|AFKU|                      --------------------------------------------------


gi|1854587|gb|U32964.1|LPU3296      GTTTAGTTTTGAGGGGATCACCCTCAAGCACCCTARCGGGTGCGACTTTG 100
gi|5902977|gb|AF121200.1|           GTTTAGTTTTGAGGGGATCACCCTCAAGCACCCTAACGGGTGCGACTTTG 98
gi|KU|gb|AFKU|                      -----------GGGGATCACCCTCAAGCACCCTARCGGGTGCGACTTTG 38
                                               ********************** **************


gi|1854587|gb|U32964.1|LPU3296      TTCTTTGAAAACTGGATATCATTGTATTAATTGTTTTAAATTGCCGAGAA 150
gi|5902977|gb|AF121200.1|           TTCTTTGAAAACTGGATATCATTGTATTAATTGTTTTAAATTGCCGAGAA 148
gi|KU|gb|AFKU|                      TTCTTTGAAAACTGGATATCATTGTATTAATTGTTTTAAATTGCCGAGAA 88
                                    ************************************************


gi|1854587|gb|U32964.1|LPU3296      CACAGCGTATTTGTATGAGTTTCTGAAAAAGAAATTCGCATCGCATAACC 200
gi|5902977|gb|AF121200.1|           CACAGCGTATTTGTATGAGTTTCTGAAAAAGAAATTCGCATCGCATAACC 198
gi|KU|gb|AFKU|                      CACAGCGTATTTGTATGAGTTTCTGAAAAAGAAATTCGCATCGCATAACC 138
                                    ************************************************


gi|1854587|gb|U32964.1|LPU3296      GCTGACGCAAGTCAGTACAGG 221
gi|5902977|gb|AF121200.1|           GCTGACGCAAGTCAGTACA-- 217
gi|KU|gb|AFKU|                      GCTGACGCAA---------- 148
                                    **********
```

DISCUSSION

Morphological, physiological and biochemical identification of collected test organism

The isolated bacteria were grown on MRS selective media. Addition of 0.05% cysteine in MRS media increased the specificity for isolation of *Lactobacillus* spp. (**Hoque *et al*., 2010; Hartemink *et al.,* 1997**). Small, rounded, creamy, white colony and rod-shaped cells further support our claim on morphological background. The isolate was found to be gram positive and at the same time, endospore, catalase and motility negative, summing it up to be a *Lactobacillus* sp. These characteristics can be correlated with **Hoque *et al.* (2010)**, **Islam *et al.* (2012)** and **Honi *et al.* (2013)**.

Extraction of chromosomal DNA

In gel electrophoresis, extracted DNA appeared as a distinct band denoting the manufacturer's isolation protocol was mild to generate enough DNA yield. Spectrophotometer revealed good quality DNA ratio was isolated with very good yield (1010-1070 µg/ml). Quantification of DNA concentration was done at 260 nm, 280 nm and 320nm.

PCR amplification of 16s-23s rRNA gene ISR of *Lactobacillus paracasei* ssp. *paracasei*-1

In this study, in order to sequence the 16s-23s ISR rRNA gene, we have used primer pairs Lp1F and Lp1R (**Honi *et al*., 2013**). The primer pairs specifically anneal and amplify 16s-23s ISR rRNA gene when correct genomic DNA was present during the thermal cycling reaction. The PCR condition was same as described by **Honi *et al.* (2013)**. PCR amplification with the primer pairs followed by electrophoresis produced bands. **Honi *et al.* (2013)** reported an outcome of 146 bp band, using the same primer pairs Lp1F and Lp1R, which was in exact concordance to *L. paracasei* ssp.

Sequences of the 16s-23s ISR rRNA gene of *L. paracasei* ssp. *paracasei-1*

The internal spacer region of 16s-23s rRNA gene sequence of *Lactobacillus* genus is 221 bp long (**Tilsala *et al*., 1997**). Sequence signals of pGEM vector confirmed the reliability of our hardware and the protocol used in the sequencing purpose. We have obtained 148 nucleotides sequences of the 16s-23s ISR rRNA gene homologous to position no. 63 to 210 of the *L. paracasei* ssp. *paracasei-1* using PCR DNA-sequencing method. There is scope, for the future, to improve the primer design so that it can bind more upstream position of the 16s-23s ISR region of *L. paracasei* ssp. *paracasei-1*.

Comparison of 16s-23s ISR rRNA gene of *L. casei* group

There have been lots of controversies over the identification of *L. casei* group *(L. casei*, *L. paracasei and L. rhamnosus*) (**Ward *et al*., 1999**). A few species of *Lactobacillus*, including species of *L. casei* group, may be recovered from a variety of diverse habitats because of their ecological adaptability. It is empirical that bacterial genome adaptation and evolution occur via three major processes: 1) decay of genes that no longer offer a fitness benefit 2) gaining of exogenous genes or gene clusters by bacteria through horizontal gene transfer (HGT) and 3) modification of existing genes by mutation with vertical inheritance (**Broadben *et al*., 2012**). In our previous experiment (**Honi *et al.*, 2013**) we supported that the bacteria reported by **Hoque *et al.* (2010)** and **Islam *et al.* (2012)** was *Lactobacillus paracasei* ssp. *paracasei-1*. We obtained PCR amplification of 16s-23s ISR rRNA gene based on which we reported the isolate as *Lactobacillus paracasei* ssp. *paracasei-1*. Because of the taxonomic dispute and close genetic similarities, however, in this study we extrapolate that the isolate could be one of the members of *L. casei* group rather than *L. paracasei*. We wanted to confirm the identification based on nucleotide sequencing, more specifically by sequencing of 16s-23s ISR rRNA gene, which provides an accurate basis for identification (**Tannock *et al*., 1999**). In doing so, we had to compile the sequence signature of 16s-23s ISR rRNA genes of *L. casei* group obtained from the NCBI data bank (comparison 1). By comparing 16s-23s ISR rRNA gene (217 to 222 bp) across the three members of *L. casei* group we have identified thirteen signature sequence locations. **Mori *et al.* (1997)** also reported thirteen signature sequences in *L. casei* group members by comparing, however, 1.5 Kbp long 16rDNAs of *L. casei* group. The high frequency of variability between 16s-23s ISR rRNA gene could have evolved because of the higher sequence and fragment length polymorphism (**Felis *et al*., 2001**). From the determined signature sequences between these three species of *L. casei* group, we were confirmed that our isolated stain (*L. paracasei* ssp. *paracasei-1*) was *L. paracasei.*

Between *L. paracasei* (U32964.1) and *L. casei* (AF121200.1) we have observed five signature sequence for 16s-23s ISR sequence, based on *L. paracasei* numbering, chronologically which were base position no. 1, 2, 86, 220, and 221 (comparison 2). The base pair location 86, based on *L. paracasei* numbering, appeared to be a critical signature sequence for separating *L. casei* and *L. paracasei*. It suggests that in order to differentiate *L. paracasei* from *L. casei,* based on 16s-23s ISR rRNA gene sequencing, we have to confirm the base pair of location no. 86. When we blasted the sequence of 16s-23s ISR rRNA gene of *L. paracasei* ssp. *paracasei-1* with strain U32964.1 (*L. paracasei*) and AF121200.1 (*L. casei*) we have found only difference at position no 86 (comparison 3). In *L. paracasei* ssp. *paracasei-1* sequence it was R (A or G) which matched with *L. paracasei* strain. The result of comparison-3 (*L. paracasei* ssp. *paracasei-1*, *L. casei* and *L. paracasei*) reveals that our isolate was *L. paracasei* ssp *paracasei-1*.

CONCLUSION

Our study shows that comparison of the percentages of similarity between 16s-23s ISR rRNA gene sequences of lactobacilli provides a practical method of strain identification. The small 16s-23s ISR rRNA gene spacer sequences of lactobacilli are around 200 bp in length. These relatively short sequences can be easily sequenced on both polynucleotide strands and provide reliable information for comparative bacterial identification research. Moreover, sequencing of the spacer region restrains the advantage in distinguishing *Lactobacillus* spp from the members of *L. casei* group which cannot be accomplished by comparison of 16S V2-V3 region sequences. The findings of this research is a very strong bolstering evidence for the research works of **Islam *et al.* (2012)** and **Honi *et al.* (2013)** who also identified the bacteria, in different approaches though, as *L. paracasei*

ssp. *paracasei-1*. We suggest that while differentiating between *L. caesi* and *L. paracasei,* the base position no. 86, based on *L. paracasei* numbering, can solve the identification dilemma. Purine (R) base at position no. 86 confirms the strain as *L. paracasei* while pyrimidine (Y) base confirms the bacteria as *L. casei*. Similarly, it can be suggested that other closely related bacterial group, as like *L. casei* group, can be separated from the group members by sequencing 16s-23s ISR rRNA gene because of the higher sequence and fragment length polymorphism. The series of our research activities (**Hoque *et al.*, 2010; Islam *et al.*, 2012** and **Honi *et al.*, 2013),** including this one, can be utilized in screening potential probiotic bacteria for food and pharmaceuticals application.

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EFFECT OF LOW TEMPERATURE STORAGE ON THE THERAPEUTIC PROPERTIES OF THE IMMOBILIZED ON ADSORBENTS AND FREE CELLS OF *SACCHAROMYCES BOULARDII*

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ABSTRACT

Probiotic *S. boulardii* cells were immobilized on adsorbents "Sorbex" (activated carbon pellets) and "SCMS-1" (granules of aluminum dioxide with deposited film of activated carbon). The experimental probiotics were the "carrier-cells" systems, wherein on the carrier surface different amounts of yeast cells were absorbed. The safety of the "carrier-cells" systems was assessed on the number of macrocolonies formed by certain systems. During freezing down to low temperatures bigger number of immobilized cells if compared to free cells in the cell suspensions has been shown to dye. The safety of the complexes during freezing is affected by cooling mode and preserving medium composition. Experimental intestinal dysbiosis was induced in mice by oral administration of ampicillin and metronidazole. There has been found a longer persistence of yeast cells and higher levels of *Bifidobacterium spp*., *Lactobacilus spp.* bacteria in the colon mucin of the mice treated with immobilized *S. boulardii*. In the groups of animals treated with free cells and the mixtures of free cells with adsorbents, the terms of persistence of yeast cells and the recovery rates of *Bifidobacterium spp., Lactobacilus spp.* in colon mucin were lower. Storage for one year at –80°C, –196°C did not affect therapeutic properties of immobilized and free *S. boulardii* cells.

Keywords: Dysbiosis, probiotics, adsorbents, immobilization, low temperatures

INTRODUCTION

The "intestinal dysbiosis" term is considered as a symptom, testifying to quantitative and/or qualitative disorders in indigenous parietal intestinal microflora (**Vorobyov *et al*., 1998**). Here the dysbiosis is defined as more general notion, indicating a microbiological imbalance in the body. Intestinal dysbiosis is an aggravating factor in clinical pathology of various origins and the functioning disorders of different organs and systems (**Tkachenko and Suvorov, 2009**). The range of therapeutic measures to correct the dysbiotic changes in the gut includes pathogenetic treatment of the main disease, if necessary it also comprises a selective contamination of pathogenic and opportunistic microorganisms, restoring indigenous parietal intestinal microflora (**Ardatskaya, 2008; Tokareva, 2011**).

To correct the indigenous microflora there are applied various probiotic products: probiotics, prebiotics, synbiotics, metabiotics, probiotic dietary supplements (**Bondarenko, 2012**; **Guarner *et al*., 2012**). A number of randomized controlled trials and meta-analyzes of the experts of the Cochrane Collaboration shows that with the help of probiotic drugs a specific therapeutic or prophylactic effect in many pathologies can be achieved. A positive effect of probiotics was reported in the recommendations and communications of the FAO, WHO, WGO (**Hempel *et al*., 2012**;**Andreeva,2006; Floch *et al*., 2011; Floch, 2014; Probiotics,2012**; **Kailasapathy, 2002**).

However, in some cases, no clinical effect of using the probiotics has been found. Many researchers believe that negative results when using probiotics are associated either with the death of probiotic microorganisms while passing through the natural protective gastrointestinal barriers or with biological incompatibility of the administered probiotic cells and patient's intestinal indigenous flora (**Darmov *et al*., 2011**). To increase the effectiveness of probiotic drugs there are designed the new formulations of those, namely the immobilized probiotics. The immobilized probiotics in microcapsules and gel carrier (**Ding and Shah, 2009; Chen *et al*., 2007**) have become more widespread. The studies on immobilization of probiotics on the carriers are poor.

The research aim was to study the effect of immobilized on adsorbents probiotic *Saccharomyces boulardii*, stored at low temperatures, on recovery of parietal microflora of *Bifidobacterium spp*., *Lactobacilus spp.* in the animals with experimental intestinal dysbiosis.

MATERIALS AND METHODS

Research object

The study was performed with *Saccharomyces boulardii (S.boulardii)* yeast species. Yeasts were isolated from the commercial drug "Enterol" ("Biocodex», France). Yeasts were grown on Sabouraud agar medium (**Polyak *et al*., 2008**) for 48 hrs at a temperature of 30 ± 2°C. For further manipulations the cells were washed-out from the medium surface with 0.14 M NaCl solution.

Immobilization

The adsorbents "Sorbex" ("Ecosorb", Ukraine) and "SCMS-1" ("Novosibirskfarm", Russia) were experimentally selected as the carriers. Adsorbent "Sorbex" represents the granules of activated carbon, "SCMS-1" adsorbent is aluminum dioxide granules, coated with the activated charcoal film. Both of them have the developed meso- and macroporous structures. Yeast cells were immobilized on the carriers as described (**Vysekantsev *et al*., 2011**). Into a 500 ml flask there was added 200 ml of cell suspension in 0.14M NaCl solution with a concentration of $5 \cdot 10^7$ cells/ml. To the suspension there was added 1g adsorbent. The flask was shaken for 30-60 min at a temperature of 0 ÷ 2°C. Free and non-immobilized *S. boulardii* cells served as the control.

Freezing

Before freezing the preparations of free and immobilized cells were pelleted by centrifugation and re-suspended in 5% sucrose solution. Free cell suspension and supernatant of the "carrier-cells" systems were introduced into "Corning" cryovials ("Corning Incorporated", USA) with a 1.8 ml handling volume. Part of the samples was cooled with a «Cryoson» programmable freezer for bioobjects (Germany) down to –40°C with 1°C/min cooling rate, and then immersed into liquid nitrogen. The second part of the samples was placed on the shelves of low-

temperature chamber “Jouan VX 380” (France) with a temperature of –80°C. The samples were stored for a year at –196 and (–80 ± 4)°C respectively. The samples were thawed in a 30°C water bath.

Survival examination

The viability of free cells was examined by Koch dish method (**Lusta and Fichte, 1990**) on the colony formation of Sabouraud agar. To test the effect of low temperature storage on the immobilized cell preparations we have used the term "preservation rate", i.e. the number of macrocolonies forming the individual "carrier-cells" systems. The preservation rate of the systems was examined by our own method (**Martsenyuk *et al.*,2012**). The need of this method applying and its layout stages could be found in «Results and discussion» section.

Scanning electron microscopy

Scanning electron microscopy (SEM) was performed with a scanning electron microscope REMMA 101 A («Selmi», Ukraine). The preparations for electron microscopy were fixed in 2.5% glutaraldehyde solution with 0.1 M Na-cacodylate buffer, were dehydrated in ethyl alcohol. After dehydration the preparations were silver sprayed.

Luminescent microscopy

For fluorescence microscopy there was used a confocal laser scanning microscope LSM 510 META («Carl Zeiss», Germany). Dead cells were propidium iodide (PI) stained («Sigma-Aldrich», USA) as described **(Dang, 2011**). PI concentration in aliquots was increased from 3 up to 5 μg/ml.

Therapy of experimental dysbiosis with immobilized *Saccharomyces boulardii*

The experiments were performed in 7-8 months-old white random-bred mice weighing of 18-20 g. All the manipulations were carried out in accordance with General Principles of Experiments in Animals (**European, 1986**). The animals were etherized for anesthesia. To simulate an intestinal dysbiosis the animals were orally administered using a gastric tube with 5 mg ampicillin ("Arterium", Ukraine) and 2 mg metronidazole ("Unique Pharmaceutical Laboratories", India) for 3 days. Within 48 hrs after administration of ampicillin and metronidazole to the animals there was started the treatment with the preparations of free *S. boulardii* cells, mixtures of free and immobilized cells. The treatment lasted 12 days. Probiotics and adsorbents were administered once a day. The daily dose of free cells was $1.6 \cdot 10^4$ cells. Taking into account the safety of the systems of immobilized cells after low temperature storage the dose was increased twice. To days 5, 12 of dysbiosis therapy and 5, 10 days later the treatment in the animals a colon mucin was sampled, in mucin there was examined the content of the number of *Bifidobacterium* and *Lactobacillus* bacteria (**Ivanov *et al*., 2002**) as well as of *Saccharomyces boulardii* yeasts. To isolate the yeasts serial mucin dilutions were plated on Sabouraud agar medium supplemented with chloramphenicol ("Arterium", Ukraine), at a final concentration of 0.05 g/l (**Polyak *et al*., 2008**). Mucin preparations for light microscopy were Gram and Giemsa-Romanovsky stained (**Korolyuk and Sboychakov, 2002**). For additional fixation of sorbent granules the surface of preparations was agar gel coated on a phosphate buffer solution with pH 7.2 – 7.4 (**Morozov, 1999**). Mucin preparations were examined using “Zeiss Primo Star" microscope (Germany) with the “Axio Vision 4" software.

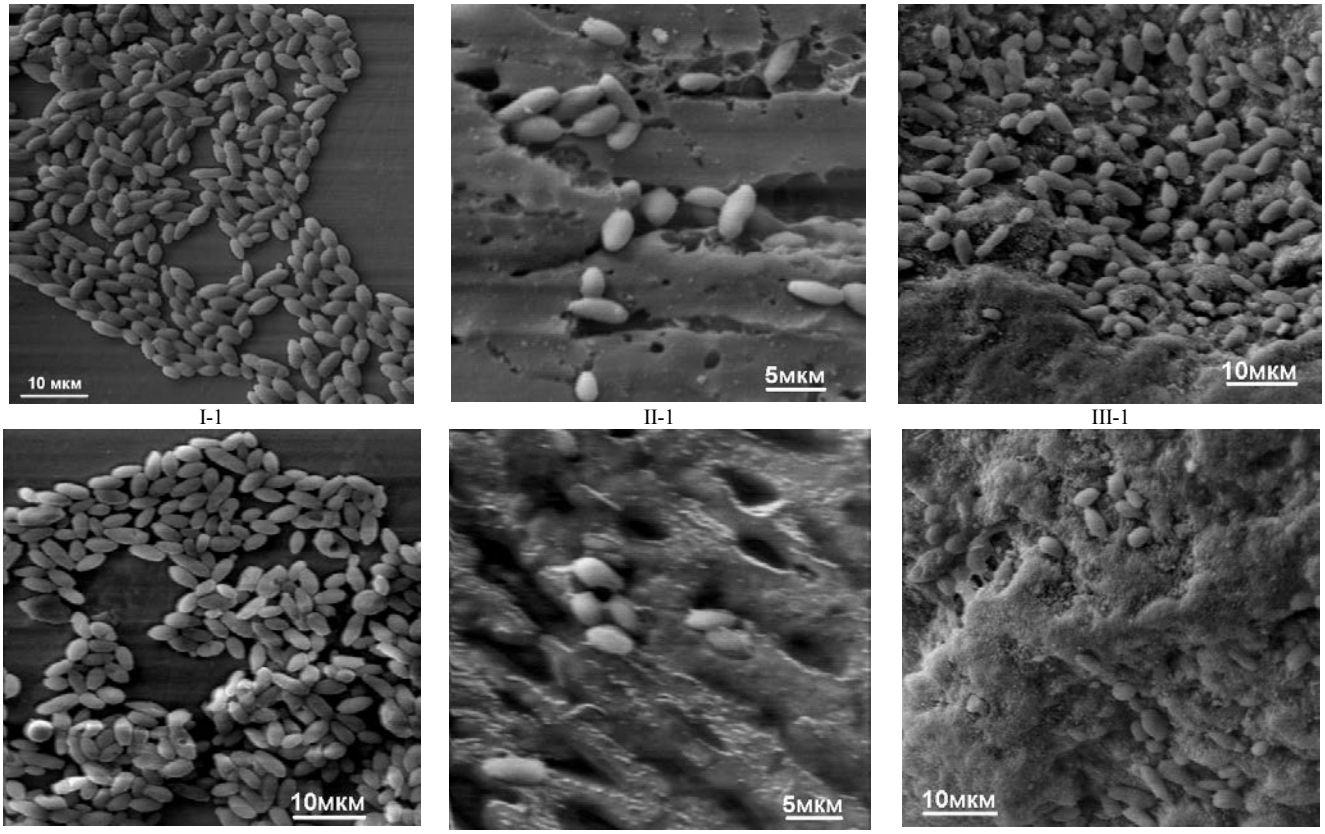
Statistical analysis of the results

For statistical analysis of the findings the software package SPSS 17.0 («IBM», USA) was used. Statistical error threshold was set at 5%.

RESULTS AND DISCUSSION

Preservation rate of the “carrier-cell” systems after freezing and storage

The first step was to study the post-thaw safety of the "carrier-cells" systems. The authors of research papers and patents dealing with the immobilization of probiotics on the carrires, determined the preservation of probiotic drugs either on cumulative effect on the composition of luminal intestinal microflora of the animals with experimental dysbiosis, or on the number of macrocolonies of probiotics after seeding by Koch dish method (**Lusta and Fichte, 1990**). Both methods used do not objectively assess the safety of namely the "carrier-cells" systems. The first method is mediated one on biological effects of drugs. Second one provides the overestimated results. When using it the authors considered the macrocolonies, formed by the "carrier-cells" systems and free cells, which were not immobilized or desorbed from the carrier surface during rehydration or thawing.



I-1 II-1 III-1

I-2 II-2 III-2

Figure 1 1 Electronograms of preparations of free (I) and immobilized on «Sorbex» (II) and «SCMS-1» (III) *S.boulardii* cells prior to (1) and after freezing down to –196°C (2).

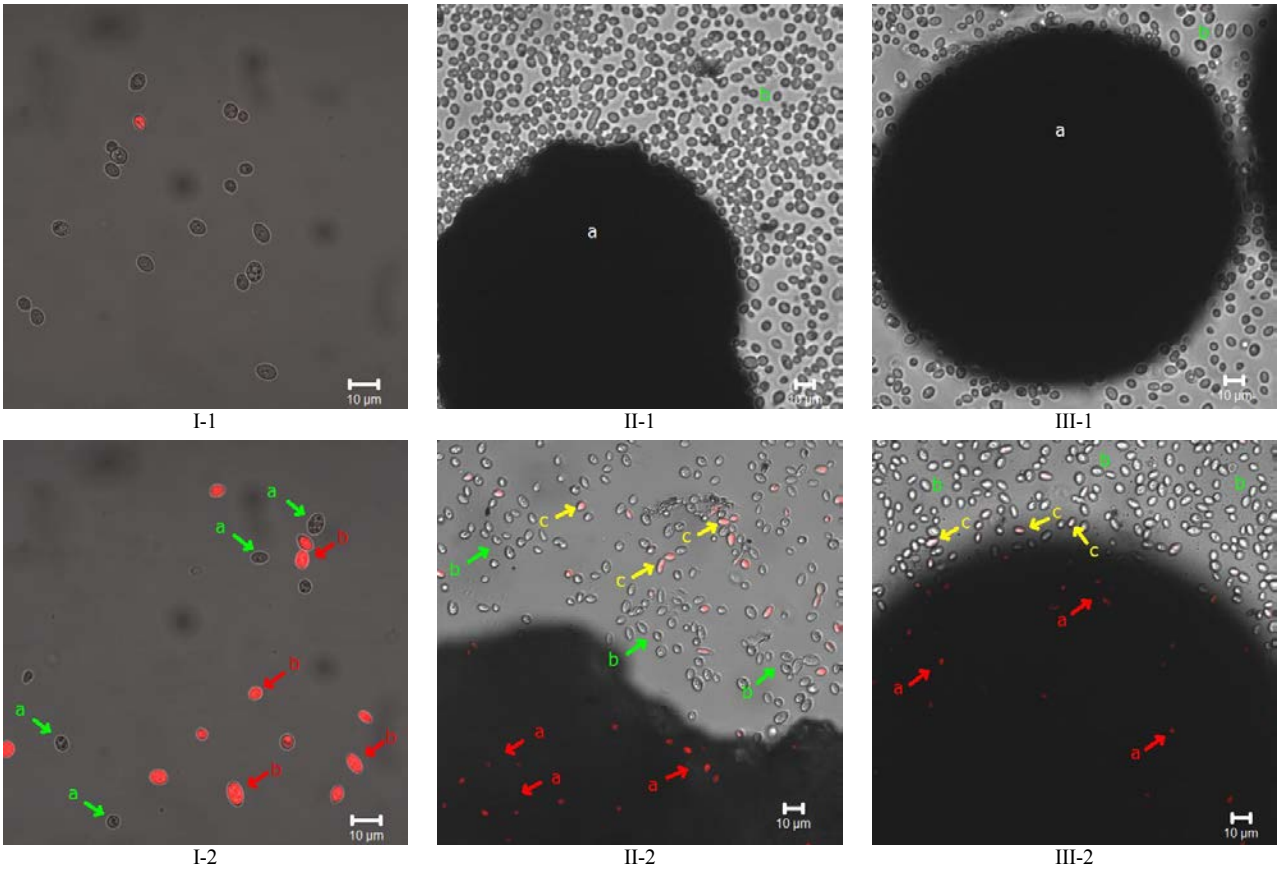


Figure 2 Confocal laser scanning microscopy of preparations of free (I) and immobilized on «Sorbex» (II) and «SCMS-1» (III) *S.boulardii* cells prior to (1) and after freezing down to –196°C (2): a – immobilized cells with PI inclusion; b – intact free cells; c – free cells with PI inclusion.

This analysis was confirmed by our research results for the preparations of immobilized *S. boulardii* cells using SEM and confocal fluorescence microscopy. In the photomicrographs obtained by the SEM, a portion of the yeast cells adhered directly to the carrier surface and the part of the cells penetrated into various depths of the carrier material macropores (Fig.1) are seen. The amount of cells in various sites of carriers differs. By outward appearances it is impossible to distinguish living and dead cells. The photomicrographs obtained by confocal fluorescence microscopy show that around "carrier-cells" systems there are free and non-immobilized cells. Among free and immobilized on the carriers' surface cells there are the ones with preserved outer structure, but with the PI inclusion (Fig.2). The number of cells with PI inclusion in different samples varies.

We have developed a method of accounting the macrocolonies formed only by the "carrier-cells" systems separated from free cells (**Martsenyuk *et al.*,2012**). In accordance with this method, the suspension of the immobilized cells is placed into a centrifuge tube with prefabricated membrane. Membrane wells allow only free cells. The tube is centrifuged, then the "carrier-cells" systems are shaken from a membrane into the tube with a certain volume of 0.2% agar. Consecutive serial dilutions in 0.2% agar solution are performed. From the serial dilutions the suspension of the complexes by 1 ml is placed into Petri dishes and poured with agar growth medium (2% agar). After solidification of agar medium the Petri dishes are cultured in an incubator and then the number of macrocolonies formed by the complexes is counted. The preservation rate for the systems is expressed by the number of colony forming units (CFU) per 1 ml supernatant or 1g adsorbent.

After storage of free and immobilized *S. boulardii* cells for 1 year the following results were obtained. At –80°C free cells did not die for 6 months. In 12 months, the number of viable cells decreased from 7.7 down to 7.0 lg CFU/ ml (Fig.3). At –196°C using the aforementioned cooling regimen free cells did not die during a year.

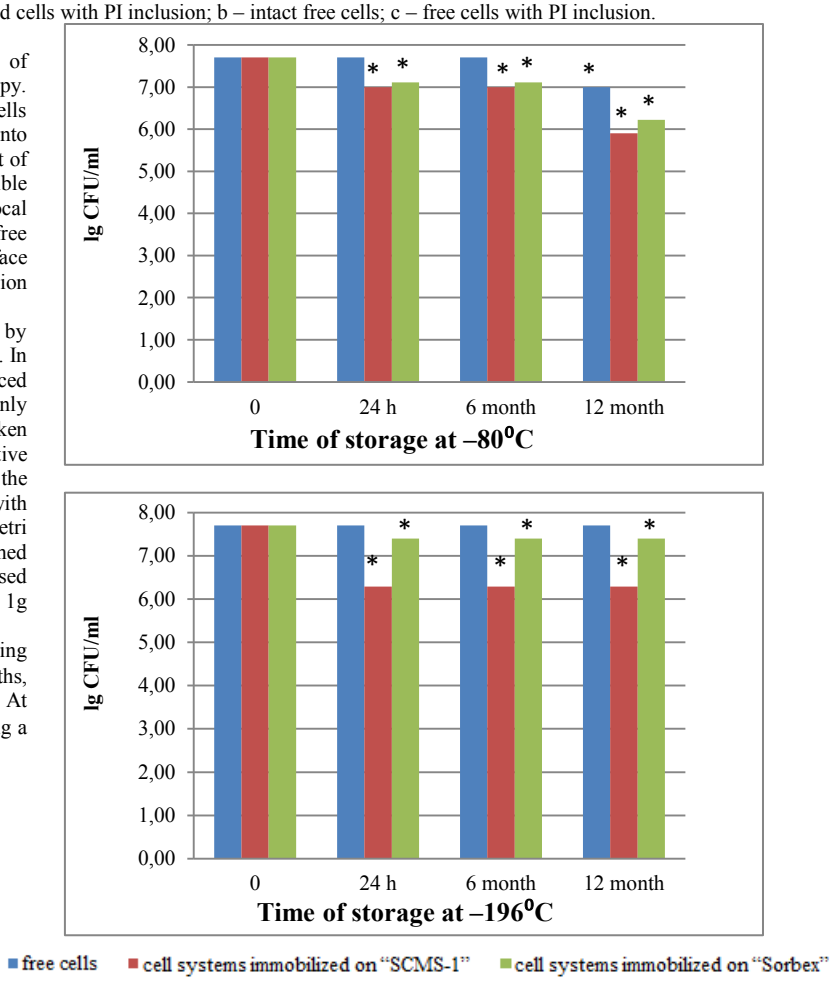


Figure 3 Preservation rate of free *S. boulardii* cells and "carrier-cells" systems after storage at – 80° and – 196°C. * – statically significant differences between initial control and experiment.

At –80°C the number of systems of the cells immobilized on adsorbent "Sorbex" decreased through day from 7.7 to 7.11 lg CFU/ml and remained at this level for 6 months. In 12 months, the safety of the systems has decreased to 6.22 lg CFU/ml. Number of the systems of the cells immobilized on "SCMS -1" adsorbent in 24 hrs also reduced from 7.7 down to 7.0 lg CFU /ml, remained at this level for 6 months, and in 12 months decreased to 5.9 lg CFU / ml.

After freezing down to –196°C the number of the systems with "Sorbex" adsorbent decreased from 7.7 down to 7.4 lg CFU/ml, that with adsorbent "SCMS-1" reduced from 7.7 to 6.29 lg CFU/ml. During storage at –196°C for a year no extra death was observed.

Therapy of experimental dysbiosis with immobilized *Saccharomyces boulardii*

After dysbiosis induction in all the groups of animals there were noted the symptoms of intestinal dysbiosis: polifekalia, alternating diarrhea and constipation, change in stool consistency, reduction in body weight by (3.5±0.5). In 48 hrs after the last administration of chemodrugs the content of *Bifidobacterium spp*. in colon mucin decreased from (7.6±0.5) lg CFU/g down to (2.1±0.4) lg CFU/g. Content of *Lactobacillus spp*. in mucin decreased from (8.0±0.4) lg CFU/g to (2.4±0.5) lg CFU/g. *Saccharomyces spp*. yeasts were absent in mucin before and after dysbiosis induction.

In groups of the animals treated with native and stored for a year at –80, –196°C free *S. boulardii* cells and the mixtures of free cells with adsorbents (groups 4–9) to day 5 of treatment in the colon parietal mucin the *S. boulardii* concentration was from (3.7±0.3) to (4.1±0.3) lg CFU/g. To day 12 of treatment in mucin of the animals of these groups there were found from (3.9±0.3) to (4.4±0.3) lg CFU/g of yeast cells. In 5 days after the treatment the concentration of *S. boulardii* in mucin made from (0.5±0.02) to (0.9±0.02) lg CFU/g. In 10 days after the treatment there were no yeast cells in mucin of the animals of groups 1–9. In groups of the animals treated with immobilized on adsorbents *S. boulardii* cells (group 10–15), the concentration of yeast cells in mucin to day 5 of treatment was (6.0 ± 0.5) to (6.7 ± 0.5) lg CFU/g at day 12 of treatment from (6.5±0.4) to (6.8±0.4) lg CFU/g. In 5 days after the treatment the concentration of yeast cells in mucin of the animals of these groups ranged from (5.9±0.46) to (6.2±0.35) lg CFU/g and in 10 days it was of (2.8±0.4) to (3.1±0.4) lg CFU/ g.

Storage for one year at –80, –196°C did not affect the ability of *S. boulardii* to persist in the colon mucin of the mice with experimental dysbiosis.

In all the groups of *S. boulardii* treated animals there was noted an increase in the *Bifidobacterium spp*., *Lactobacilus spp*. populations. In the mucin of the animals treated with free *S. boulardii* cells (groups 1–3), to day 12 of treatment the amount of *Bifidobacterium spp*., *Lactobacilus spp*. increased to (3.3±0.4) – (3.5±0.5) lg CFU/g, 10 days after the end of treatment it was up to (4.3±0.3) – (4.5±0.5) lg CFU/g (Table 1). In mucin of the animals treated with a mixture of free *S. boulardii* cells with adsorbents (groups 4–9) the number of *Bifidobacterium spp.* increased up to (3.8±0.3) – (3.9±0.4) to day 12 and up to (4.9±0.3) – (5.0±0.5) lg CFU/g in 10 days later the treatment. In mucin of the animals treated with the preparations of immobilized *S. boulardii* cells (groups 10–15), the amount of *Bifidobacterium spp.* in mucin to day 12 of treatment was (4.1±0.4) – (4.3±0.4), and 10 days later the end of treatment this was (5.3±0.3) – (5.5±0.4) lg CFU/g.

The number of *Lactobacilus spp.* in mucin of the animals treated with free *S. boulardii* cells, increased to day 12 of treatment to (4.1±0.3) – (4.3±0.3) lg CFU/g, in 10 days after treatment it was up to (4.8±0.4) – (4.9±0.5) lg CFU/g. When applying the mixtures of *S. boulardii* cells with enterosorbents the number of *Lactobacillus spp.* to day 12 of treatment increased up to (4.5±0.4) – (4.6±0.4), and 10 days after the treatment it was up to (5.2±0.3) – (5.4±0.3) lg CFU/g. In mucin of the animals treated with immobilized *S. boulardii* cells, the amount of *Lactobacillus spp*. in mucin to day 12 of treatment was (4.9±0.4) – (5.1±0.4), and 10 days later the treatment ceasing it was (5.9±0.3) – (6.2±0.5) lg CFU/g.

Storage for one year at –80 and –196°C did not affect the ability of free and immobilized *S. boulardii* cells to contribute to a rise in *Bifidobacterium spp*. and *Lactobacilus spp*. populations in colon mucin.

Table 1 Content of *Bifidobacterium spp.* and *Lactobacillus spp.* in large intestine parietal mucin of mice with chemotherapeutic intestinal dysbiosis after introduction of free and immobilized yeast cells and adsorbents

Groups of animals (introduced preparations)	**Number of lg CFU/г, $\bar{X} \pm S\bar{x}$**							
	Bifidobacterium spp.				*Lactobacillus spp.*			
	Prior to dysbiosis induction	In 48 hrs after dysbiosis induction (first 24 hrs of observation)	Day 12 of the receiving *S.boulardii* preparation (observation day 12)	In 10 days after terminating the receiving of *S.boulardii* preparations (observation day 22)	Prior to dysbiosis induction	In 48 hrs after dysbiosis induction (first 24 hrs of observation)	Day 12 of the receiving *S.boulardii* preparation (observation day 12)	In 10 days after terminating the receiving of *S.boulardii* preparations (observation day 22)
Animals with no treatment	7.6±0.5	2.1±0.4	2.8±0.3	3.6±0.4*	8.0±0.4	2.4±0.5	2.3±0.3	3.0±0.4*
1. Free *S.boulardii* cells			3.5±0.5	4.3±0.4*			4.2±0.4	4.8±0.4*
2. Free *S.boulardii* cells stored at –80°C			3.3±0.4	4.3±0.3*			4.1±0.4	4.8±0.4*
3. Free *S.boulardii* cells stored at –196°C			3.3±0.5	4.5±0.5*			4.3±0.3	4.9±0.5*
4. Free *S.boulardii* cells + «SCMS–1»			3.8±0.4	4.9±0.3*			4.5±0.4	5.2±0.3*
5. Free *S.boulardii* cells + «Sorbex»			3.8±0.5	4.9±0.4*			4.6±0.4	5.3±0.3*
6. Free *S.boulardii* cells stored at –80°C + «SCMS–1»			3.8±0.3	4.9±0.3*			4.5±0.4	5.4±0.4*
7. Free *S.boulardii* cells stored at –80°C + «Sorbex»			3.8±0.3	5.0±0.5*			4.5±0.3	5.3±0.3*
8. Free *S.boulardii* cells stored – 196°C + «SCMS-1»			3.9±0.4	4.9±0.3*			4.4±0.3	5.3±04*
9. Free *S.boulardii* cells stored at –196°C + «Sorbex»			3.8±0.5	4.9±0.4*			4.5±0.4	5.4±0.3*
10. Immobilized on «SCMS–1» *S.boulardii* cells			4.1±0.4	5.3±0.3*			4.9±0.4	5.9±0.3*
11. Immobilized on «Sorbex» *S.boulardii* cells			4.2±0.3	5.3±0.4*			4.9±0.4	6.0±0.4*
12. Immobilized on «SCMS–1» *S.boulardii* cells stored at –80°C			4.3±0.4	5.3±0.3*			4.9±0.4	5.9±0.3*
13. Immobilized on «Sorbex» *S.boulardii* cells stored at – 80°C			4.3±0.4	5.4±0.3*			4.5±0.3	5.9±0.3*
14. Immobilized on «SCMS–1» *S.boulardii* cells stored at –196°C			4.1±0.5	5.5±0.4*			5.0±0.4	6.0±0.4*
15. Immobilized on «Sorbex» *S.boulardii* cells stored at –196°C			4.3±0.3	5.4±0.3*			5.1±0.4	6.2±0.5*

Notes:

* – $p<0.05$ if compared with the content of bacteria (*Bifidobacterium spp., Lactobacillus spp.*) to day 12 of therapy and 10 days after terminating the receiving of *S.boulardii* preparations.

During microscopy of the mucin preparations for the animals of all the groups to day 12 of treatment and 5, 10 days after the treatment there was observed a rise in cell numbers of lactobacilli and bifidobacteria. Yeast cells in mucin of the animals of groups 1–9 was found to day 5 after the treatment end. In mucin of the animals of groups of 10–15 treated with immobilized *S. boulardii*, to day 10 after the treatment end there were single free yeast cells and sorbent granules with no cells and with immobilized yeast cells.
In the groups of animals treated with immobilized probiotics to day 10 after the treatment end there were no clinical signs of intestinal dysbiosis. In other groups we observed some clinical manifestations of dysbiosis.

DISCUSSION

The findings indicate the research prospects on creating the probiotics, immobilized on sorbents. The developed method for assessing the preservation rate of the "carrier-cells" systems isolated from free cells allows to objectively estimate the safety of viable cells immobilized on the surface of the carrier particles. It was for the first time shown that the sensitivity of yeast cells immobilized on the carrier surface to damaging effects of freeze-thawing was higher if compared with free cells.
On the basis of generally accepted statements of the two-factor theory of cell cryoinjury (**Mazur, 1967; Mazur *et a*l., 1972**), we assume the following reasons of higher death rate of the immobilized cells. A part of the surface of the immobilized cells closely contacts with the sorbent structures. The volume of the cells is herewith maintained, but the surface of cells through which the water transport out of the cells at the stage of extracellular crystallization beyond the cells decreases. This strengthens an intracellular crystallization at the determined cooling rate. A second reason may be the damages of cell wall and cytoplasmic membrane on the boundary zone of the recorded on the cell surface carrier sites when reducing the cell volume during the transport of water out of them.
Due to the mentioned above to efficiently store the immobilized on adsorbents probiotics the choice of cooling mode and composition of preserving medium is crucial.
We used the conditions of low temperature storage of the immobilized on carbon-containing adsorbents *S. boulardii* probiotic ensured the safety of therapeutic doses required for a growth stimulation of parietal populations of *Bifidobacterium spp.*, *Lactobacilus spp*. in mice with experimental intestinal dysbiosis.
The results of the studies performed indicate that for efficient influence of probiotics their engraftment to a biofilm as part of gut microbiota is not mandatory. *S.boulardii* yeasts do not belong to microbiota of homoiothermal animals and are not able to adhere to an intestinal mucosa, but the clinical effect of their use is shown in several studies (**Vandenplas *et a*l., 2009**). In our research we found that the introduction of free *S. boulardii* cells, the mixtures of adsorbents with free cells and those immobilized on adsorbent boosted the parietal populations of *Bifidobacterium spp*., *Lactobacilus spp*. This was due to the creation of the conditions ensuring the reproduction of indigenous bifidobacteria and lactobacilli which remained in the intestines of the animals after the dysbiosis induction in the concentrations below critical. An attention is drawn to the fact of more pronounced therapeutic effect of immobilized *S. boulardii*. The most likely explanation for this is as follows. The "carrier-cells" systems are cell conglomerates formed by co-adhesion. After an adhesion of the "carrier-cells" systems to an intestinal mucosa there are formed the microcolonies with the following structure: yeast cells - adsorbent granule - mucin – mucus epithelium. Herewith in the discrete sites of mucous with the mentioned microcolonies there is the number of yeast cells which is sufficient for stimulation of the reproduction of indigenous bifidobacteria and lactobacilli by their metabolites. This is supported by higher concentrations and longer persistence of yeast cells in mucin of the animals, which were injected with immobilized *S. boulardii*.
In the research we used the preparations of the "carrier-cells" systems, purified from free cells to study the effect of the systems *per se*. When developing the commercial drugs of probiotics immobilized on sorbents there is no need to purify them from free cells.

CONCLUSION

The idea of this study was to improve the ability of transient probiotic *S. boulardii* to stimulate the recovery of indigenous microflora (*Bifidobacterium spp., Lactobacilus spp*.) in the animals with a modeled chemotherapeutic intestinal dysbiosis. It was experimentally shown that the preparations obtained by immobilizing the yeast cells on the granules of carbon-containing adsorbents caused the pronounced therapeutic effect when treating an intestinal dysbiosis in animals if compared to the drugs of free cells and the mixtures of free cells with adsorbents. In the animals treated with immobilized probiotic *S. boulardii*, parietal *Bifidobacterium spp*., *Lactobacilus spp*. populations were restored more intensively and clinical manifestations of intestinal dysbiosis disappeared quickly. Low temperature storage (–80, –196°C) provides a high preservation rate of free and immobilized on adsorbents *S. boulardii* cells. The results can be used to develop new probiotic drugs and design the drug delivery systems.

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BACTERIA ASSOCIATED WITH NON-ALCOHOLIC FERMENTED BAMBOO SHOOT FOOD PRODUCT

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ABSTRACT

Pure bacterial cultures were isolated from fermented products collected from two locations of North East India (Assam and Arunachal Pradesh). Forty four variants were identified by 16S rRNA gene sequencing. The dominant microbial genera found were *Bacillus, Paenibacillus* followed by *Oceanobacillus* and *Lactobacillus* in Assam and *Bacillus, Enterococcus, Lactobacillus* in Arunachal-Pradesh food products. Bacillus isolates showed extracellular enzyme production (amylases, proteases and lipases) as analyzed through plate assay. GC-MS analysis showed beneficial chemical components like organic acids, esters, aminoacids, vitamins in fermented bamboo shoot products. Probiotic attributes of culturable bacterial isolates from bamboo fermentations gives promiscuity for human consumption. Beneficial microorganisms from bamboo shoot fermented food products of North-Eastern region of India were explored and compared using bioinformatics tools.

Keywords: Non-alcoholic fermentation, bamboo shoots, bacteria

INTRODUCTION

Bamboo shoots as food products are rich source of various macro and micro nutrients but needs processing before consumption. Fermentation is one of the processing method which increases the nutritional quality of the bamboo food product in terms of its amino acids, sugars, esters and organic acid contents **(Jeyaram *et al*., 2008)**. Although fermentation is one of the oldest and most economic methods of food preservation and ethnic people of Sub-Himalayan region of North East India, especially Assam and Andhra Pradesh prepare and consume a variety of domesticated and wild bamboo tender shoots and their fermented products, there is need to increase the probiotic attributes to the food for betterment of health **(Jeyaram *et al*., 2008, Qureshi *et al*., 2014)**. Some popular fermented bamboo tender shoots of North East India are Mesu of Sikkim, Arunachal Pradesh, Henoop, Khorisa of Assam, Hikung ,Mesu, Bastenga, Hiring of Arunachal Pradesh, Lung-siej of Meghalaya, Soibum, Soidon, Soijm of Manipur (**Tamang *et al*., 2008**). Henoop, Khorisa, Mesu, Hikung, Bastenga are traditional non-alcoholic fermented food products of Assam and Arunachal Pradesh consumed by Kharbi, Sonowal, Assamese community of Assam and Monpa, Nepali community of Arunachal Pradesh of North-East India (**Das *et al*., 2012; Tamang *et al*., 2012**). Fermented food products are not only rich in nutrients such as proteins, vitamins, essential amino acids, sugars, fatty acids but are also good for digestion (**Jeyaram *et al*., 2008, Das *et al*., 2012**).
Most of the bacterial species associated with the fermented food do not possess health risk, they are designated as GRAS (generally recognized as safe) organism (**Hansen, 2002, Das *et al*., 2012**). The objective of our study was to demonstrate culturable microbial diversity of traditionally processed fermented bamboo shoot products of Assam and Arunachal Pradesh, India using molecular approach and designate probiotic attributes of each isolate as safe and healthy food for consumption.

MATERIAL AND METHODS

Total six food samples of fermented bamboo shoot products were collected from different parts of Assam and Arunachal Pradesh in India. Out of six, three samples were from Erdangte, Khowang, North-Lakimpur, Dibrugarh locations of Assam and three samples were from Ziro, Bomdila, Bhalukpung of Arunachal Pradesh. All the samples were collected aseptically into sterile containers and transferred to the laboratory for analysis. . Fermented bamboo shoot products were prepared by defoliating and finely chopping the young edible shoots of the bamboo plant. The small pieces of bamboo shoots were placed tightly into green hollow bamboo stem and were covered with leaves of bamboo. This was then allowed to ferment at room temperature for 7-14 days. These fermented bamboo shoots were extensively used as pickles, curries and as additives in various recipes

Characterisation of fermented bamboo shoot products

Microbiological analysis

Each food product (5gms) was homogenized with 45ml of 1X PBS. It was diluted serially in the same diluents. Appropriate decimal dilution (100µl) of the homogenate was spread over different media plates such as Man, Rogosa and Sharpes (MRS) agar plate (Himedia), nutrient agar, 30 different media plates having different media components and incubated at 30° C for 24-48 hrs. Morphologically different colonies were selected and pure cultured by streaking repeatedly on respective agar media plates. The isolates were grown in respective broth and pure cultures were preserved in 30% glycerol at -80°C (Sanyo, Ultralow deep freezer). The average number of microbes present per gram of different types of samples were also calculated, which was expressed in terms of CFU (colony forming units) per gram of sample (Table 1).

GC-MS based metabolite profiling of fermented bamboo shoot products

Sample preparation was carried out by modifying the method described earlier by various researchers (**Ojinnaka *et al*., 2013, Lee *et al*., 2007**). Fermented bamboo shoot products were finely ground in mortar and 500mg of the grinded samples were extracted with 5ml of methanol at 60°C in a heated water bath for 30 min. Samples were cooled to ambient temperature for 30 min and then vortexed for 30-60seconds prior to centrifugation at 3000rpm, at 4ºC for 10 min. Supernatant obtained was passed through the oven-dried anhydrous sodium sulfate. The filtrate obtained were used for GC-MS analysis.
GC-MS analysis (GC Clarus 500 Perkin Elmer system) of a food sample each from Assam and Arunachal-Pradesh was performed employing the following conditions: column Elite-1 fused silica capillary column (30 x 0.25 mm ID composed of 100% Dimethylpolysiloxane), operating in electron impact mode at 70 eV; helium (99.999%) was used as carrier gas at a constant flow of 1 ml /min and an injection volume of 0.5 µl was employed (split ratio of 10:1) injector temperature 250ºC; ion-source temperature 280ºC. The oven temperature was programmed from 150ºC (isothermal for 2 min), with an increase of 6ºC/min, to 200ºC, then 5ºC/min to 300ºC, ending with a 10 min (isothermal at 300ºC). Mass

spectra were taken at 70 eV; a scan interval of 0.5 seconds and fragments from 40 to 450 Da. Total GC running time of 50min. The GC-MS data was interpreted for identification of various components by comparing their retention time and mass spectra with the known component stored in the database of National Institute Standard and Technology (NIST) library having more than 62,000 patterns. The name, molecular weight and structure of the components of the test materials were ascertained (Table 2).

Total dna extraction from pure bacterial culture

The method used for the total genomic DNA extraction of 176 isolates was the modification of the method of (**Jeyaram *et al.*, 2010; Kapley *et al*., 2000**). A single colony from the agar medium was inoculated to 5ml of respective broth and incubated overnight at 30^{o} C in respective conditions. Culture volume of 1 OD at 600nm absorbance was measured (UV-1800 Shimadzu UV/Vis Spectrophotometer) and centrifuged at 8000rpm for 10min (Hettich Model: Mikro 220R, Germany). The cell pellet was washed twice with sterile distilled water (Cascada Bio water, Pall life science) and finally resuspended in 100ul of TE buffer (Sigma) with 10µl of lysozyme (1mg/ml) (Sigma). The cell suspension was incubated at 37^{o} C for 30min in constant temperature water bath (Cyber Lab) with intermediate mixing. It was then allowed to cool at room temperature. 25µl of 0.5 M NaOH was added. It was mixed gently and was incubated at room temperature for 30 min. Then 25µl of Tris pH 7.5 was added to it for neutralization. Finally the 345µl of sterile Milliq water was added to make up the volume to 500 µl. The content was gently mixed and allowed the debris to settle at the bottom at room temperature or at 4^{0}C. Then the supernatant was carefully pipette out and used for PCR analysis either immediately or can be stored at -20^{0} C until required.

The variation in bacterial strains was studied by RAPD (Random Amplified Polymorphic DNA) analysis. Two primers (RAPD 58, RAPD 59) were used after screening several RAPD Primers. A 50 µl reaction mixture consisting of 5 µl of cell free lysate with 50ng DNA, 5 µl of 10X PCR Buffer, 3 µl of 25mM MgCl2, 5 µl of 2.5µM primer(IDT), 2 µl of dNTPs, 0.5 µl of Taq (5U/ µl) (Applied Bioscience).The PCR reaction was carried out in Veriti thermal cycler (Applied Biosystem) through following temperature cycles: temperature profile starts with initial denaturation cycle of 5min at 95^{0} C followed by 35 cycles each consisting of denaturation at 94^{0}C for 1min, annealing at 40^{0} C for 1 min, extension at 72^{0}C for 2minutes with final extension of 72^{0}C for 10min. followed by cooling to 4^{0}C. The number of bands and their migration pattern was analyzed by agarose gel (1.2%) electrophoresis. The gels were documented using gel documentation system. The biotypes of isolates were determined based on DNA band profiling. On the basis of RAPD profiles the bacterial strains showing highly similar banding patterns were grouped together and the strains showing different banding patterns were selected for sequencing.

Identification of bacterial bsolates by 16s rDNA sequence

16S rDNA PCR of the selected representative strains of each RAPD group were performed with universal primer 27F (5'AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3'). PCR was performed using the 50 µl reaction mixture consisting of 50-100ng of crude DNA, 5 µl of 10X PCR buffer,3 µl of 25mM $MgCl_2$, 2 µl of 2.5µM primer (IDT), 2ul of dNTPs, 0.5 µl of Taq (5U/ µl) under the following conditions: 4 min initial denaturation at 95°C; 35 cycles of denaturation (30s at 95°C), annealing (1 min at 55°C), and extension (2 min at 72°C); a final extension at 72°C for 10 min. PCR product was purified using 1% agarose gel electrophorsis. DNA fragments were manually eluted using QIAquick PCR Purification kit (QIAGEN, Germany) and sent for sequencing using primer 27F. The resulting trimmed sequenced data were compared using BLASTN to those in the NCBI nucleotide Database. The 16S rDNA sequences of representative strains of each RAPD group were aligned using Clustal X Version 2.0. with reference strains sequences obtained from NCBI database for identifying closest strain and phylogenetic relationships (Table 3 and 4).

Enzyme assay and functional diversity analysis

The purified agar medium supplemented with 10% casein and spirit blue agar supplemented with tributyrin (0.3% lipase substrate). Inoculated plates were allow to grow for 24-48 h. the clear zone around the colonies indicate positive results for protease and lipolytic activity respectively. For amylase activity, bacterial cultures were spotted on starch agar plates and incubated for 24-48 h before being flooded with iodine solution. Production of amylase was indicated by the existence of a clear zone around the colonies, while the rest of the plates stained blue-black.

Phylogenetic tree construction

All the sequences were compared with 16S rRNA gene sequences available in the GenBank databases by BLASTn search. Evolutionary analyses were conducted in MEGA5.2.2 (**Tamura *et al*., 2011**). Multiple sequence alignments of partial 16S rRNA gene sequences (≥500 bp) were carried out using CLUSTAL W (**Larkin *et al*., 2007; Thompson *et al*., 1997**). Phylogenetic trees were constructed in MEGA5.2.2 software from evolutionary distances obtained using Neighbor Joining and Maximum Composite Likelihood method. The robustness of the phylogeny was tested by bootstrap analysis using 1000 iterations. Accession numbers of all isolates can be viewed in respective phylogenetic tree.

RESULTS AND DISCUSSION:

Characterization of fermented bamboo food samples from assam and arunachal pradesh, India

In the present study, six fermented bamboo shoot products collected from different locations of North-East India, three each of Arunachal Pradesh and Assam were used for exploring the diverse population of bacteria. In each fermented bamboo shoot products the total viable bacterial count were in the range of 10^4 cfu g-1 to 10^5 cfu g-1 of sample (**Table.1**).

Table 1 Table showing total viable bacterial count, pH and water activity from food samples of Assam and Arunachal Pradesh

Location	Local Name	pH	Total viable Bacterial count cfu/g Sample	Water activity (aW)
Assam	Henoop	4.5±0.2	$3.5x10^4$	0.661
	Khorisa 1	3.8±0.1	$3x10^4$	0.595
	Khorisa 2	3.6±0.2	$8x10^4$	0.556
Arunachal Pradesh	Mesu	4.5±0.1	$6.8x10^5$	0.598
	Hikung	4.3±0.1	$2.8x10^5$	0.600
	Bastenga	4.8±0.2	$3x10^5$	0.588

The mean pH of the food sample ranged from 3 to 4.2 with Khorisa2 showing the lowest pH value (Table 1). This indicated that the fermented bamboo shoot products which we analyzed were acid fermented (**Tamang *et al*., 2008**). The water activity (a_w) of the fermented bamboo shoot products were found to be in the range of 0.556 to 0.661. Less water activity signifies less possibility of food-spoilage caused by food poisoning organisms and food-borne pathogens which requires higher water activity (a_w) for their growth (**Oyewole & Isah, 2012**). Protein and carbohydrate content of the fermented bamboo shoot products were found to be less than that of dry bamboo shoot indicating the presence of proteolytic and amylolytic activity during fermentation process. Also carbohydrates and proteins served as nutrients for bacterial biomass growth during fermentation.

Metabolite profiling of fermented bamboo shoots using gc-ms analysis

The fermented bamboo shoot samples of Assam and Arunachal-Pradesh were found to contain organic acids as dominant constituents followed by esters, amino acids and other components (**Table 2**).

Organic acids: The common organic acids in the fermented bamboo shoot products of Assam and Arunachal-Pradesh were acetic acid, hexadecanoic acid and octadecanoic acid found at retention time of 2.75min, 39.9min, and 34.5min respectively. Also butanoic acid, propanoic acids were found common in both food samples. It has been reported that butanoic acid, propanoic acid, tetrabutyl phenyl acetate compounds were determined as major aroma compounds in Korean soy sauces and barley bran sauces (**Lee *et al*., 2006; Choi *et al*., 2007; Steinhaus & Schieberle, 2007**). These compounds were used as flavor and fragrance agents in various food industries; they also provide resistance to food spoilage microorganism and thus help in preservation of food products.

Esters: During fermentation process esterification of alcohols with fatty acids leads to formation of esters. Methyl phenyl ester, linoleic acid ethyl ester, octadecanoic acid methyl ester, palmitic acid vinyl ester, ethyl esters, 2-oxo methyl ester were found to be present in the fermented bamboo shoot products of Assam and Arunachal-Pradesh. These esters are known to contribute in the characteristics pleasant aromatic and sugary flavor to the fermented food samples (**Qin & Ding, 2007; Ojinnaka & Ojimelukwe, 2013**).

Amino acids: Cystine, arginic acid, arginine, alanine, aspartic acid, aspargine, alanine were the free amino acids found in the fermented food samples. The presence of free amino acids contributes to the taste and nutritive quality of the fermented bamboo shoot products.

Other compounds found to be present in the fermented bamboo shoots are D-mannitol, β-carotene, olyl alcohol etc. which increases the nutritional quality of food (**Table 2**). Enzymatic saccharification and production of mannitol, sorbitol, xylitol etc carried out by amylase pro ducing Lactic acid bacteria have been reported in various studies (**Lee *et al*., 2012**).

Table 2 GC-MS analysis of metabolites from fermented bamboo shoot product.

	ASSAM	ARUNACHAL PRADESH
	Metabolites	**Metabolites**
Organic acids	Acetic Acid	Acetic acid
	3-Deoxy-D-Mannonic acid	N-Hexadecanoic acid
	Ethyl Malonic acid	Octadecanoic acid
	N-Hexadecanoic acid	β-carboline-3-carboxylic acid
	Octadecanoic acid	Adenosine-3-phosphoric acid
	3-Methyl Butanoic acid	
	Propanoic acid	
Esters	4-Methyl phenyl ester	4-Methyl phenyl ester
	Linoleic acid ethyl ester	9,12 Octadecanoic acid methyl ester
	9,12 Octadecanoic acid methyl ester	
	Palmitic acid vinyl ester	
	2-Oxo methyl ester	
	ethyl ester	
Amino acids	Aspargine	Arginnic acid
	Alanine	Cystine
	Glycine	
	Aspartic acid	
Other	2-3 Butanediol	Propylene glycol
	2-Amino 3-hydrooxy pyridine	2-3 Butanediol
		β-Carotene
		D-Mannitol

Table 3 Bacteria identified by 16S rDNA sequence analysis from fermented bamboo shoot products of Assam and Arunachal Pradesh

Sample Location	**16S rDNA sequence analysis**			
	Bacterial Isolates	**Accession No.[a]**	**Identification[b]**	**Similarity(%)**
Assam:	HPCAQKh1-12a	KC713926	*Bacillus amyloliquefaciens*	99%
	HPCAQKh2-25b	KC713918	*Bacillus flexus*	99%
	HPCAQKh2-23a	KC713922	*Bacillus flexus*	98%
	HPCAQKh1-4b	KC899351	*Bacillus licheniformis*	99%
	HPCAQH24d	KC713912	*Bacillus sp.*	99%
	HPCAQKh2-8c	KC899350	*Bacillus thuringiensis*	100%
	HPCAQKh2-27b	KC713917	*Brevundimonas sp.*	99%
	HPCAQH3a	KC713915	*Lactobacillus brevis*	99%
	HPCAQKh3-M6	KF574823	*Lactobacillus brevis*	100%
	HPCAQKh1-23c	KC713927	*Lactobacillus plantarum*	100%
	HPCAQKh2-25a	KC713919	*Oceanobacillus oncorhynchi*	99%
	HPCAQKh2-24c	KC713920	*Oceanobacillus sp.*	99%
	HPCAQKh2-24a	KC899349	*Paenibacillus cineris*	99%
	HPCAQKh2-12a	KC713924	*Paenibacillus cineris*	99%
	HPCAQKh2-3a	KC713916	*Paenibacillus favisporus*	99%
	HPCAQKh2-12b	KC713923	*Paenibacillus favisporus*	99%
	HPCAQKh2-11a	KC713925	*Paenibacillus favisporus*	99%
	HPCAQH24b	KC713913	*Paenibacillus sp.*	94%
	HPCAQH23d	KC713914	*Paenibacillus sp.*	96%
	HPCAQH-17a	KC899352	*Pseudomonas aeruginosa*	99%
	HPCAQKh2-23b	KC713921	*Staphylococcus pasteuri*	100%
Arunachal Pradesh:				
	HPCAQM-6d	KC899354	*Bacillus amyloliquefaciens*	100%
	HPCAQM-10b	KC899357	*Bacillus amyloliquefaciens*	100%
	HPCAQHi-2a	KC899363	*Bacillus amyloliquefaciens*	100%
	HPCAQHi-6b	KC899365	*Bacillus amyloliquefaciens*	99%
	HPCAQM-1c	KC899353	*Bacillus licheniformis*	99%
	HPCAQM-23b	KC899362	*Bacillus licheniformis*	99%
	HPCAQM-25d	KC899359	*Bacillus methylotrophicus*	98%
	HPCAQHi-5a	KC899364	*Bacillus methylotrophicus*	99%
	HPCAQM-25a	KC899360	*Bacillus methylotrophicus*	99%
	HPCAQM-8a	KC899355	*Bacillus sp.*	99%
	HPCAQM-5	KF574822	*Bacillus sp.*	99%
	HPCAQHi-7c	KC899366	*Bacillus subtilis*	99%
	HPCAQHi-23b	KC899368	*Bacillus subtilis*	99%
	HPCAQHi-14a	KC899367	*Bacillus tequilensis*	99%
	HPCAQM-8	KF574830	*Enterococcus casseliflavus*	99%
	HPCAQM-13	KF574826	*Enterococcus casseliflavus*	100%
	HPCAQM-14	KF574827	*Enterococcus casseliflavus*	100%
	HPCAQM-15	KF574828	*Enterococcus casseliflavus*	100%
	HPCAQM-1	KF574825	*Lactobacillus brevis*	99%
	HPCAQM-2	KF574829	*Lactobacillus brevis*	99%
	HPCAQM-16	KF574824	*Lactobacillus rhamnosus*	99%
	HPCAQM-24b	KC899361	*Paenibacillus dendritiformis*	99%
	HPCAQM-11b	KC899358	*Staphylococcus pasteuri*	99%

Legend [a] Accession numbers of the sequences obtained after submitting the partial 16s sequences of bacterial isolates to NCBI Genebank . [b] Identification obtained on NCBI BLAST analysis of 16S rDNA sequence of bacterial isolates

Bacterial identification

Based on morphological examination a total of 140 variable bacterial colonies from six fermented bamboo shoot products were isolated at random. Based on the RAPD DNA profiling and phenotypic variations 21 strains from fermented bamboo shoot products of Assam and 23 from Arunachal Pradesh were selected for further study. Total 44 isolates were subjected to 16S rRNA gene sequencing and identified by BLAST analysis. 16S rRNA gene sequencing and BLAST analysis of selected strains revealed that the bacteria present in the fermented food samples of Assam belongs mainly to genera *Bacilli, Lactobacilli, Staphylococcus, Oceanobacilli, Paenibacilli, Brevundimonas* and that of Arunachal Pradesh belongs to *Bacillus, Staphylococcus, Lactobacillus, Enterococcus, Paenibacillus* (**Table. 3**).

It was also observed that all six food samples were predominated by Gram-positive bacteria, with the most abundant phylum being Firmicutes **(Table 4)**. In earlier studies, dominant species associated with fermented bamboo shoots were identified as *Lactobacillus brevis. L Lactobacillus lactis, Lactobacillus fallax, Lactobacillus plantarum* (**Tamang et al., 2008, Qureshi *et al*., 2014**). Our study revealed the presence of *Paenibacillus cineris, Staphylococcus pasteuri, Oceanobacillus oncorhynchi, Paenibacillus favisporus, Brevundimonas sp*. along with *Lactobacillus brevis and Lactobacillus plantarum*. The fermented bamboo shoot products of Assam showed the presence of *Bacillus* and *Paenibacilus* as the predominant bacterial genus followed by *Lactobacillus*. Presence of *Bacilli and Paenibacilli* were also reported in fermented cassava, maize, peanuts (**Namrata *et al*., 2004**). Also reports of presence of *Bacillus licheniformis* being a predominant species found in fermented Cauim are available (**Almeida *et al*., 2007**). Our finding also showed presence of *Bacillus licheniformis* with probiotic attributes (Table 5) in both Assam and Arunachal Pradesh food samples.

Table 4 Taxonomic distribution of culturable bacterial isolates associated with fermented bamboo shoots of Assam and Arunachal Pradesh.

Phylogenetic group	Class	Genus	A(21)	AP(23)	Species	Assam	Arunachal Pradesh
						(strains)	(strains)
Firmicutes	*Bacillus*	*Bacillus*	*6*	*14*	*B. amyloliquefaciens*	*HPCAQKh1-12a*	*HPCAQM-6d*
					B. amyloliquefaciens		*HPCAQM-10b*
					B. amyloliquefaciens		*HPCAQHi-2a*
					B. amyloliquefaciens		*HPCAQHi-6b*
					B. flexus	*HPCAQKh2-25b*	
					B. flexus	*HPCAQKh2-23a*	
					B. licheniformis	*HPCAQKh1-4b*	*HPCAQM-1c*
					B. licheniformis		*HPCAQM-23b*
					B. methylotrophicus		*HPCAQM-25d*
					B. methylotrophicus		*HPCAQM-25a*
					B. methylotrophicus		*HPCAQHi-5a*
					B. sp. (bataviensis)	*HPCAQH24d*	
					B. sp. (tequilensis)		*HPCAQM-8a*
					B. subtilis		*HPCAQHi-7c*
					B. subtilis		*HPCAQHi-23b*
					B. tequilensis		*HPCAQHi-14a*
					B. thuringiensis	*HPCAQKh2-8c*	
					Bacillus sp.		*HPCAQM-5*
		Enterococcus		*4*	*E. casseliflavus*		*HPCAQM-8*
					E. casseliflavus		*HPCAQM-13*
					E. casseliflavus		*HPCAQM-14*
					E. casseliflavus		*HPCAQM-15*
		Lactobacillus	*3*	*3*	*L. brevis*	*HPCAQH3a*	*HPCAQM-1*
					L. brevis	*HPCAQKh3-M6*	*HPCAQM-2*
					L. plantarum	*HPCAQKh1-23c*	
					L.rhamnosus		*HPCAQM-16*
		Oceanobacillus	*2*		*O. oncorhynchi*	*HPCAQKh2-25a*	
					O. sp.	*HPCAQKh2-24c*	
		Paenibacillus	*7*	*1*	*P. cineris*	*HPCAQKh2-24a*	
					P. cineris	*HPCAQKh2-12a*	
					P. dendritiformis		*HPCAQM-24b*
					P. favisporus	*HPCAQKh2-3a*	
					P. favisporus	*HPCAQKh2-12b*	
					P. favisporus	*HPCAQKh2-11a*	
					P. sp.	*HPCAQH24b*	
					P. sp.	*HPCAQH23d*	
		Staphylococcus	*1*	*1*	*S. pasteuri*	*HPCAQKh2-23b*	*HPCAQM-11b*
Proteobacteria	*Alpha Proteobacteria*	*Brevundimonas*	1		*B. sp.*	HPCAQKh2-27b	
	Gamma Proteobateria	*Pseudomonas*	1		*P. aeruginosa*	HPCAQH-17a	

Legend :Number indicates number of isolates assigned to particular genus.
: Number in bracket indicates number of isolates identified from fermented Bamboo shoots of Assam(A) and Arunachal Pradesh(AP)

The presence of *Lactobacilli* in bamboo shoot food samples were responsible for acidic fermentation. *Lactobacillus plantarum,* a known facultative heterofermenter and *Lactobacillus brevis,* an obligate heterofermenter produces acetic acid along with lactic acid, were found to be present in food sample of Assam and Arunachal-Pradesh. The results indicated that these food samples were mixed acid fermented product. Also the pH values of these food samples were in acidic range (Table. 1). The production of both the end products can prevent a food from spoilage and extend the shelf life. Acidic fermentations generally offer cost effective methods of preserving food for people in developing countries, where more sophisticated means of preservation are unaffordable and could not be used.

Screening of isolates for extracellular enzymes by plate assay

Strains of *Bacilli, Staphylococcus, Lactobacilli, Oceanobacilli, Paenibacilli, Brevundimonas,* and *Enterococcus* were screened for their extracellular amylase, protease and lipase activity. A total of 19 isolated strains i.e. 7 from Assam and 12 from Arunachal Pradesh showed their ability to hydrolyze starch on agar plate, 20 isolates were found to show protease activity and 17 isolates showed lipase activity.

Among isolated strains of Assam, *Brevundimonas sp. HPCAQKh2-27b, Paenibacillus favisporus HPCAQKh2-12b, Lactobacillus plantarum HPCAQKh1-23c*, and *Bacillus amyloliquefacian HPCAQKh1-12a* showed highest amylolytic activity and from Arunachal Pradesh, *Bacillus licheniformis HPCAQM-1c, Bacillus amyloliquefaciens HPCAQM-10b,* showed highest amylolytic activity (**Table. 5**). Amylolytic strains of bacterial genera *Lactobacillus plantarum*, *Bacillus licheniformis* were also isolated from Brazilian fermented food (**Almeida *et al*., 2007**) and Nigerian fermented food (**Johansson *et al*., 1995; Sanni 2002**). The amylolytic activity was responsible for hydrolysis of starch during Bamboo shoot fermentation.

Table 5 Amylase, Protease and Lipase activity of bacterial species isolated from fermented bamboo shoot products of Assam and Arunachal Pradesh.

Location	*Bacterial Isolates*	Amylase	Protease	Lipase
Assam	*Bacillus amyloliquefaciens HPCAQKh1-12a*	++	-	-
	Bacillus flexus HPCAQKh2-23a	-	+	-
	Bacillus flexus HPCAQKh2-25b	-	+	-
	Bacillus thuringiensis HPCAQKh2-8c	+	+++	+
	Brevundimonas sp. HPCAQKh2-27b	++	-	+
	Lactobacillus brevis HPCAQH3a	-	++	+
	Lactobacillus plantarum HPCAQKh1-23c	++	+	-
	Paenibacillus cineris HPCAQKh2-12a	+	-	-
	Paenibacillus favisporus HPCAQKh2-12b	++	++	++
	Paenibacillus favisporus HPCAQKh2-3a	+	-	-
	Pseudomonas aeruginosa HPCAQH-17a	-	-	+
Arunachal Pradesh				
	Bacillus amyloliquefaciens HPCAQHi-6b	++	++	+
	Bacillus amyloliquefaciens HPCAQHi-2a	++	+++	+
	Bacillus amyloliquefaciens HPCAQM-1-b	+++	+++	++
	Bacillus amyloliquefaciens HPCAQM-6d	++	+++	+
	Bacillus licheniformis HPCAQM-1c	+++	++	-
	Bacillus licheniformis HPCAQM-23b	+	+	+
	Bacillus methylotrophicus HPCAQHi-5a	++	+++	+
	Bacillus methylotrophicus HPCAQM-25a	+	+	+
	Bacillus methylotrophicus HPCAQM-25d	-	++	-
	Bacillus sp. HPCAQM-8a	++	++	+
	Bacillus subtilis HPCAQHi-23b	+	++	+
	Bacillus subtilis HPCAQHi-7c	++	++	+
	Bacillus tequilensis HPCAQHi-14a	++	+++	+
	Enterococcus casseliflavus HPCAQM13	-	-	+
	Staphylococcus pasteuri HPCAQM-2b	-	+	-

Legend: + ─ zone of clearance ≤ 10mm
++ ─ zone of clearance >10mm ≤ 15mm
+++ ─ zone of clearance > 15mm

Proteolytic enzyme assay revealed that there was appreciable difference in protease activity among the strains identified. The protease secreting ability of genus *Bacillus* have been well known (**Nascimento & Martin 2004; Beg and Gupta 2003**) and we found total six strains showing highest protease activity indicated by a zone of clearance of more than 150mm. In general *Lactobacilli* showed low protease activity. Low protease activity of *Lactobacilli* have been reported in fish fermentation also by Namrata et al., 2004. Reports showing both amylase and protease activity of *Lactobacilli* was also available (**Thapa *et al*., 2006**).

Lipolytic activity was found comparatively less than the amylase and protease activity. Total 17 isolated strains showed liypolytic activity. *Paenibacillus favisporus HPCAQKh2-12b* strain showed highest lipolytic activity (zone of clearance more than 100mm).

Amylolytic, proteolytic and lipolytic properties of fermenting microorganism may be important for degradation of starch (which determines the availability of free sugars), proteins(which determines the availability of essential amino acids) and lipids (which determine the availability of short-chain fatty acids in particular). These properties may have considerable effects on the taste and flavor of fermented food products. Strains isolated from Arunachal Pradesh showed comparatively higher enzyme activity than that of Assam although both the regions belong to North-East India (**Table. 5**).

Bacteria isolated in this study were found to possess extracellular enzyme activity (amylases, proteases, lipases) which were responsible for the breakdown of raw organic molecules (polymers) resulting into accumulation of certain byproducts of smaller molecular weights, which may improve the organoleptic and nutritional quality of food products.

Phylogenetic analysis

Phylogenetic tree based on 16s rRNA gene sequences placed the 21 isolates of Assam into 2 major groups viz; Firmicutes and Proteobacteria and Arunachal Pradesh into single group Firmicutes. The major cluster Firmicutes were further divided into smaller sub-clusters of *Paenibacilli, Bacilli, Oceanobacilli, Staphylococcus, Lactobacilli*. The genus *Bacillus* (Firmicutes) represented the major group in food samples of both the location (**Fig 1, Fig 2**). In Assam sample dominant population was represented by the genus *Paenibacillus* followed by the genus *Bacilli* and then by *Lactobacilli* and *Oceanobacilli* while in Arunachal Pradesh food samples *Bacillus* was the only genus found to be dominant.

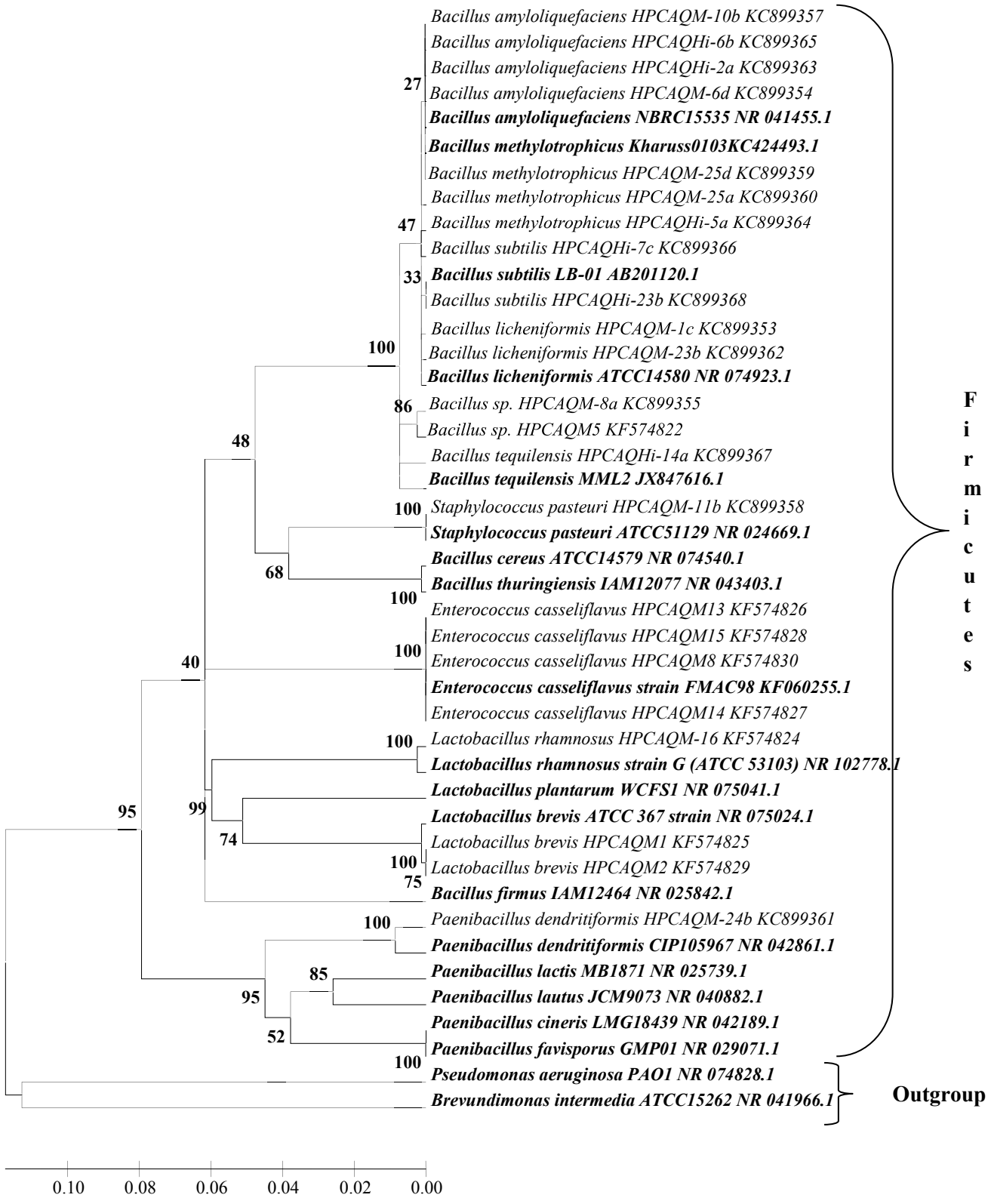

Figure 1 Phylogenetic relationship of Arunachal-Pradesh isolates: based on the partial 16S rDNA sequences of representative isolates generated using the Neighbor-Joining method in MEGA5. The reference sequences were obtained from NCBI database are indicated in bold

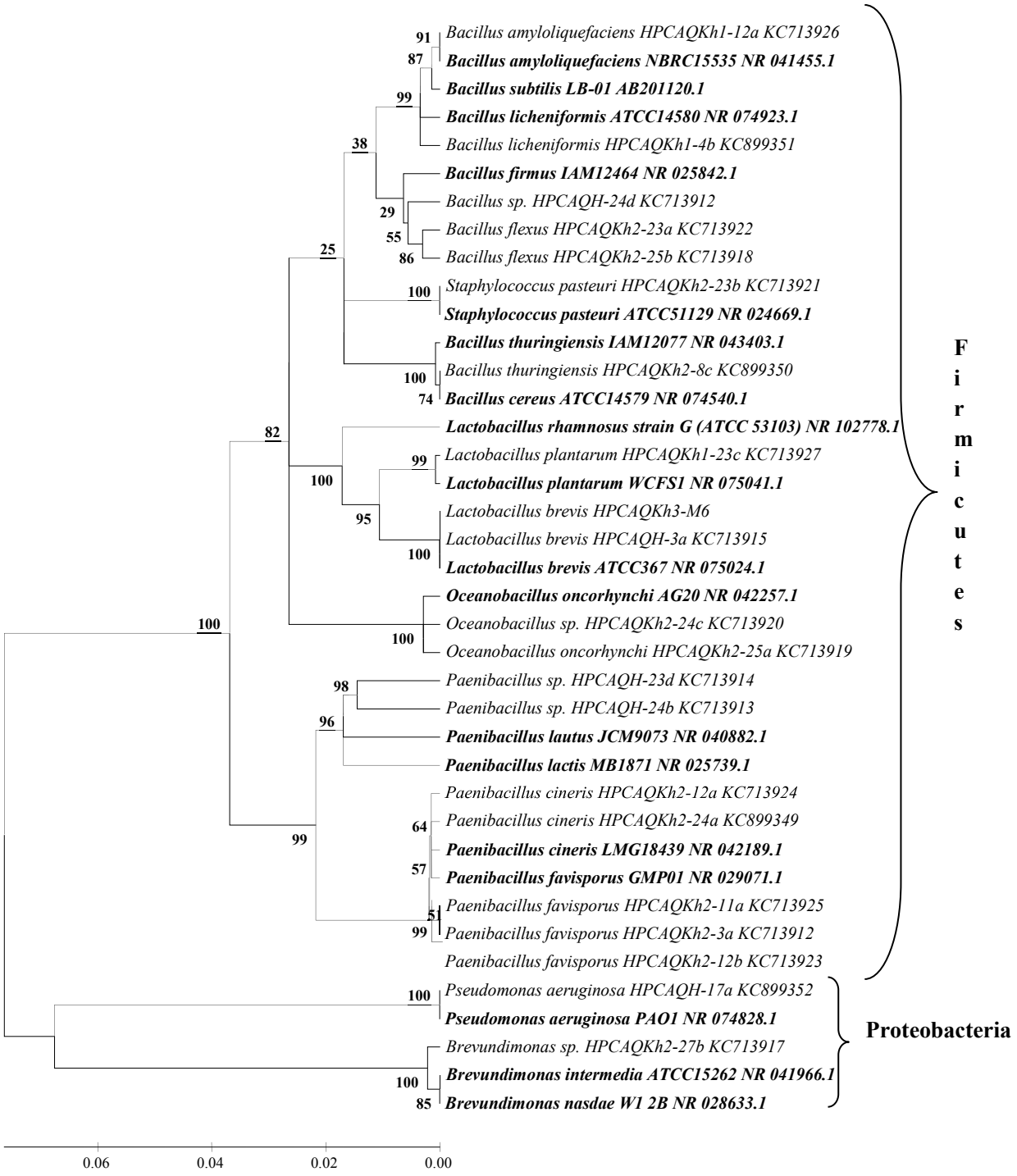

Figure 2 Phylogenetic relationship of Assam isolates: based on the partial 16S rDNA sequences of representative isolates generated using the Neighbor-Joining method in MEGA5.2.2. The reference sequences were obtained from NCBI database are indicated in bold.

CONCLUSION

The culturable bacterial diversity of non-alcoholic bamboo shoot fermented food products of North East India (Assam and Arunachal Pradesh) by using molecular tool like 16S rDNA sequencing were envisaged. The dominant microbial genera found in Arunachal-Pradesh food products were *Bacillus, Paenibacillus, Oceanobacillus* and *Lactobacillus*. However in Assam *Enterococcus* were present additionally. Mainly *Bacillus* species from both the locations showed extracellular enzyme activity (amylases, proteases and lipases) which added to the probiotic attribute of the food along with the beneficial metabolites like esters and amino acids. The results infer to provide idea for formulating the functional starter cultures for large scale bamboo fermentations for food industries.

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MYCOTOXIN-PRODUCING PENICILLIUM AND OTHER FUNGI ISOLATED FROM SLOVAK WINE GRAPES AT VARIOUS STAGES OF MATURATION

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ABSTRACT

The aim of the study was to monitor the mycobiota in grape samples at 3 maturation stages (pea berry, early veraison and ripe berry) and to test the ability of selected *Penicillium* strains to produce mycotoxins in year 2014. Grapes came from Nitra wine growing region, Šintava subregion and were analyzed by plating methods. From Devín grape variety the 339 strains were detected and identified. The most abundant genera found by descending order were *Alternaria* (42.8%), *Cladosporium* (24.2%) and *Epicoccum* (10.0%). *Penicillium expansum* was isolated from all 3 maturation stages but in low average relative density (1.2%). From Frankovka modrá (Frankish) grape variety the 562 strains were detected and identified. The most abundant genera found by descending order were *Alternaria* (30.6%), *Cladosporium* (21.3%) and *Penicillium* (20.3%). Totally were identified 8 species of the genus *Penicillium*. The most important species, on the basis of the isolation from all maturation stages and relative density, was *P. expansum*. Selected isolates of *Penicillium* species were tested for their toxigenity, according to thin layer chromatography method. In total 14 *Penicillium* strains representing 3 potentially toxigenic species were tested for their toxigenic ability, namely *P. expansum, P. griseofulvum* and *P. chrysogenum*. Out of 14 strains, 64% produced et least one mycotoxin as revealed by the method used here.

Keywords: *Penicillium*, wine grapes, maturation stages of grapes, TLC method, mycotoxins

INTRODUCTION

The grape microbial ecosystem is composed of highly diverse microorganisms, including yeasts, bacteria and fungi (**Barata *et al.*, 2012**). Molds are ubiquitous with various genera commonly found on grapes (**Rosa *et al.*, 2002**). Mold growth plays an important role in spoilage of grapes, of their pathogenicity to the harvested products, in the physical and chemical stability as well as the sensory properties of the future wine. Fungal spoilage of grapes causes substantial financial losses to growers and processors and may pose a health threat to the consumer if the contaminating fungi produce mycotoxins.

Contamination of grapes by different moulds occurs during preharvesting, harversting and grape processing. The fungal growth begins in grapes if temperature and humidity are suitable. Rotting and spoilage of grape berries before harvest can be caused by a variety of fungal species such as *Alternaria* spp., *Aspergillus* spp., *Botrytis cinerea*, *Cladosporium* spp., *Eurotium* spp., *Penicillium* spp. and *Rhizopus* spp. These genus are regarded as the main natural contaminants of grapes (**Valero *et al.*, 2005**).

Changes in the total fungal population over the growing season have been observed. At berry set, fungal populations were large in all vineyards, regardless of the grape variety, but the predominant species differed (**Bau *et al.*, 2005; Bellí *et al.*, 2006; Gomez *et al.*, 2006; Melki Ben Fredj *et al.*, 2007**). Harvest was considered to be a critical period in the growing season for fungal development. A decrease in acidity, sugar accumulation, the berry cuticle becoming brittle, damage to berries and an adaptation of mycotoxin-producing fungi to hot and humid environments may account for the abundance of fungi and the high levels of mycotoxins (**Clouvel *et al.*, 2008; Lasram *et al.*, 2012**).

It is important to identify fungal contaminants in grapes because some moulds can grow and produce mycotoxins while certain yeasts and moulds can cause infections or allergies (**Tournas and Katsoudas, 2005**). The mycotoxin production is characteristic of the species and therefore by identifying the species one can predict potential mycotoxin hazards. The major mycotoxigenic fungi that attack harvested fruits and vegetables are *Aspergillus*, *Penicillium* and *Alternaria* species – and the mycotoxins produced by them in the host tissues, e.g., aflatoxins, ochratoxin A, patulin and alternaria toxins (**Barkai-Golan, 2001**).

Penicillium species are filamentous fungi that are widely distributed in nature and can spoil food and food products (**Samson and Frisvad, 2004**). Most *Penicillium* spp. are ubiquitous soil fungi that are associated with organic matter in nature, however, their presence as epiphytes on grapes has also been reported. Some species of *Penicillium* are important plant pathogens that cause considerable economic losses in apples, citrus, grapes, and other unrelated crops in different regions of the world (**Donoso and Lattore, 2006**). For instance, *P. expansum* Link is an aggressive, destructive postharvest pathogen and a mycotoxigenic species that has been frequently reported as the cause of blue mold in stored apples and pears and causes a large part of the economic losses that occur during storage and shipment (**Barkai-Golan, 2001**). The species of *Penicillium* have gained attention as grapevine pathogens that cause blue mold decay at harvest (**Donoso and Latorre, 2006**).

The objective of the study was to identify the natural mycobiota in wine grapes collected from the Nitra wine region, with focus on genera *Penicillium*. In the vineyard, samples were taken at the pea-sized berry stage, at the beginning of berry ripening (veraison), and at harvest stage. Special emphasis was laid on the ability of some potentially toxigenic penicillia to produce some selected mycotoxins by thin layer chromatography method (TLC).

MATERIAL AND METHODS

Study area and samples

Slovak republic has 6 distinct wine-growing zones (the Small Carpathians, the Southern Slovak, the Nitra, the Central Slovak, the Eastern Slovak and the Tokaj wine regions). They spread from the west to the east of the country along its southern and south-western borders. We had samples from the Nitra wine region, Šintava subregion, village Báb. Two samples – 1 of white grape variety Devín and
1 of blue grape variety Frankovka modrá at 3 maturation stages were mycologically analyzed. One sample of a wine grape variety was represented by three subsamples of wine grapes, which were sampled in left, middle and right part of the vineyard. Samples were collected from July 2014 to October 2014, in the maturation stages corresponding to pea berry (early July), early veraison

(middle August) and harvest (middle October). The berries from the vineyards sampled were generally in good condition without visible damage. The samples of wine grapes were taken to sterile plastic bags and transported to a mycological laboratory for immediate processing.

Mycological analysis of grapes

The detection of fungi in grape samples was made by plating methods without surface disinfection. A total of 50 berries (6 - 7 healthy berries per bunch) from each sample were randomly selected and plated on Dichloran Rose Bengal Chloramphenicol agar medium (DRBC, MERCK, Germany), and incubated at 25 °C in the dark for one week. The spore-producing filamentous fungi detected were identified according to the manuals of **Samson *et al.* (2002a, 2010).** *Penicillium* strains were isolated and cultivated in MEA (Malt extract agar, **Samson *et al.*, 2010**), CYA (Czapek yeast agar, **Samson *et al.*, 2010**), CREA (Creatine-Sucrose agar, **Samson *et al.*, 2010**) and YES (Yeast Extract agar, **Samson *et al.*, 2010**) to obtain pure cultures and identify further species. Genus *Penicillium* was identified to species level based on morphological characters according to special mycological literature of **Pitt and Hocking (1997), Samson and Frisvad (2004)** and **Samson *et al.* (2002a, 2010).**

Results evaluation

The obtained results were evaluated and expressed according to relative density (RD). The relative density (%) is defined as the percentage of isolates of the species or genus, occurring in the analyzed sample (**Guatam *et al.*, 2009**). These values were calculated according to **González *et al.* (1999)** as follows:

$$RD\ (\%) = (ni / Ni) \times 100$$

ni – number of isolates of a species or genus; Ni – total number of isolated fungi.

Toxinogenity analysis

Toxinogenity of selected isolates was screened in *in vitro* conditions by means of thin layer chromatography (TLC) according to **Samson *et al.* (2002b)**, modified by **Labuda and Tančinová (2006).** Extracellular metabolites – citrinin, patulin and griseofulvin were carried out on YES agar and intracellural roquefortin C and cyclopiazonic acid on CYA agar. A few pieces of mycelium with approximate size 5 x 5 mm were cut from colonies and placed in an Eppendorf tube with 500 µL of chloroform: methanol – 2:1 (Reachem, Slovak Republic). The content of the tubes was stirred for 5 min. by Vortex Genie ® 2 (MO BIO Laboratories, Inc. – Carlsbad, CA, USA). The volume 30 µL of liquid phase of extracts along with 10 µL standards (Sigma, Germany) was applied on TLC plate (Alugram ® SIL G, Macherey – Nagel, Germany). The plate was put into TEF solvent (toluene: ethyl acetate: formic acid – 5:4:1, toluene – Mikrochem, Slovak Republic; ethyl acetate and formic acid – Slavus, Slovak Republic). After elution the plate was air-dried. Identification of the metabolites was done by comparison with metabolite standards. Roquefortin C was visible after spraying with $Ce(SO_4)_2$ x 4 H_2O as an orange spot. Cyclopiazonic acid was visible directly in daylight after spraying with the Ehrlich reagent as a violet-tailed spot. Patulin by spraying with 0,5% methylbenzothiazolone hydrochloride (MBTH, Merck, Germany) in methanol and heating at 130 °C for 8 min and then detectable as a yellow-orange spot. Directly under UV light with a wavelength of 365 nm was visualized citrinin as a yellow-green-tailed spot and griseofulvin as a blue spot.

RESULTS AND DISCUSSION

The filamentous fungi identified in white grape variety Devín from July 2014 to September 2014 by the direct plating method are indicated in Table 1. Without surface disinfection, a total of 339 strains belonging to 7 genera and *Mycelia sterilia* were identified. The three most abundant genera found by descending order were *Alternaria, Cladosporium* and *Epicoccum*. *Trichoderma* and *Penicillium expansum* were detected in more than 1% of the berries analyzed. The remaining genera were detected in less than 1% of the berries. *Penicillium expansum* can cause rot in grapes, but does not usually attack grapes before harvest (**Serra *et al.*, 2005**). *Penicillium expansum* was isolated from all 3 maturation stages but in low frequency. Also other studies have identified *P. expansum* as the species most frequently isolated from Portuguese (**Abrunhosa et al., 2001**) and French (**La Guerche et al., 2004; Bejaoui et al., 2006**) vineyards.

The mycobiota changed with maturation stage. The most frequent genera by descending order at pea berry were *Alternaria, Cladosporium* and *Epicoccum*. These constitute 79% of the fungi identified. The highest incidence of *Cladosporium* in grape was observed at this stage. *Rhizopus* occurred only at this stage. The fungal genera most frequently isolated at setting were *Alternaria* (**Bau *et al.*, 2005; Bellí *et al.*, 2006**) and *Aspergillus* (**Gomez *et al.*, 2006; Melki Ben Fredj *et al.*, 2007**). *Cladosporium* and *Penicillium* were isolated less frequently (**Bau *et al.*, 2005; Bellí *et al.*, 2006; Gomez *et al.*, 2006; Melki Ben Fredj *et al.*, 2007**).

The changes occurred in the incidence of *Alternaria, Cladosporium, Epicoccum* and *Penicillium expansum* at early veraison. *Alternaria, Epicoccum* and *Penicillium expansum* increased, while the *Cladosporium* incidence decreased. *Mucor* was detected only at this stage. The most frequent genera by descending order were *Alternaria, Cladosporium* and *Epicoccum*, representing 80.5% of the fungi identified at early veraison.

The changes occurred in the incidence of *Alternaria, Cladosporium* and *Penicillium expansum*, which decreased from early veraison to ripe berry. Our results corresponding with **Bau *et al.* (2005), Bellí *et al.* (2006), Gomez *et al.* (2006), Melki Ben Fredj *et al.* (2007**). There the percentage of grapes contaminated with *Alternaria* spp. and *Cladosporium* spp. also clearly decreased with the ripening of the grape berries. The most frequent genera by descending order were *Alternaria, Cladosporium* and *Epicoccum*, representing 71.3% of the fungi identified at this stage. *Trichoderma* occurred only at this maturation stage.

Table 1 Fungi identified in white grape variety Devín from July 2014 to September 2014 by the direct plating method

Fungi	Number of colonized berries							
	Pea berry	RD (%)	Early veraison	RD (%)	Harvest	RD (%)	Total	RD (%)
Alternaria	41	37	58	48.3	46	42.6	145	42.8
Cladosporium	37	33.3	26	21.6	19	17.6	82	24.2
Epicoccum	10	9	12	10.0	12	11.1	34	10.0
Mucor	0	-	3	2.5	0	-	3	< 1
Penicillium expansum	1	< 1	2	1.7	1	< 1	4	1.2
Rhizopus	1	< 1	0	-	0	-	1	< 1
Trichoderma	0	-	0	-	5	4.6	5	1.5
Mycelia sterilia	21	18.9	19	15.8	25	23.1	65	19.2
Total identified fungi	111		120		108		339	

Legend: RD – Relative density

The filamentous fungi identified from blue grape variety Frankovka modrá by the direct plating method are indicated in Table 2. Without surface disinfection, a total of 562 strains belonging to 11 genera and *Mycelia sterilia* were identified. The 4 most abundant genera found by descending order were *Alternaria, Cladosporium, Penicillium* and *Epicoccum. Botrytis cinerea, Fusarium* and *Trichoderma* were detected in more than 1 % of the berries analyzed. *Arthrinium, Geotrichum, Mucor* and *Rhizopus* were detected in less than 1% of the berries. **Abrunhosa *et al.* (2001**) reported that *Alternaria* and *Cladosporium* were more frequently isolated from red grape varieties than from white grape varieties, regardless of the vineyard in Portugal considered. A total of 114 *Penicillium* isolates were obtained from healthy red cultivares Frankovka modrá, 12 were obtained at pea berry stage, 23 at early veraison and 79 at ripe berry stage. Based on the results obtained, increasing *Penicillium* populations were found near harvest in agreement with studies **Latorre *et al.* (2011**) and **Díaz *et al.* (2011**), however, the overall *Penicillium* population was considerably less abundant than the populations of *Cladosporium* spp., which are epiphyte fungi commonly found on grapevines (**Latorre *et al.*, 2011**).

A total of 181 fungal isolates, representing 7 genera and *Mycelia sterilia* were identified from pea berry samples. The most frequent genera by descending order at pea berry were *Alternaria* and *Cladosporium,* which constitute 69% of the fungi identified. The highest incidence of *Fusarium* in grape, which was not detected in white grape variety Devín, was observed at this stage. Healthy cluster

of Cabernet Sauvignon grapes obtained *Penicillium* population low in Alhué and Alto Jahuel in Chile (0.006 CFU.cm^{-2} berry) at the pea-sized berry stage. They increased to 0.009 and 0.12 CFU.cm^{-2} berry at the beginning of berry ripening stage and to 0.016 and 0.013 CFU.cm^{-2} of berry at harvest in Alhué and Alto Jahuel, respectively (**Díaz *et al.*, 2011**) what corresponding with our results.

The highest number of isolates (220) represented 7 genera and *Mycelia sterilia* was detected at early veraison. The most frequent genera by descending order were *Alternaria, Cladosporium, Epicoccum* and *Penicillium*, representing 83.6% of the fungi identified at this stage. All of these fungi increased from pea berry to early veraison. The highest incidence of *Alternaria, Cladosporium, Epicoccum, Botrytis cinerea and Trichoderma* was observed at this stage.

The most frequent genera by descending order were *Penicillium*, *Epicoccum, Alternaria* and *Cladosporium* at harvest stage, representing 85.8% of the fungi identified. *Penicillium* increased, while *Alternaria, Epicoccum* and *Cladosporium* decreased from early veraison to ripe berry. The highest incidence of *Penicillium* was observed at this stage (49.1%). *Arthrinium, Geotrichum, Mucor* and *Rhizopus* occurred only at maturation stage. At harvest, the most prevalent mycobiota in four French vineyards (Alsace, Beaujolais, Bordeaux and Burgundy) were found to be *Penicillium* species (**Diguta *et al.*, 2011**). By contrast, in southern vineyards, black aspergilli have been identified as the predominant mycobiota on grapes at harvest time, although these fungi may be found on the surface of healthy grapes at all stages, regardless of the vineyard considered (**Cabañes *et al.*, 2002; Da Rocha Rosa *et al.*, 2002; Sage *et al.*, 2002; Serra *et al.*, 2003; Bellí *et al.*, 2006; Bau *et al.*, 2005; Gomez *et al.*, 2006; Guzev *et al.*, 2006; Leong *et al.*, 2006**). The genus *Aspergillus* is a component of the epiphytic flora of grapes and may be present on grape berries. The surface of infected berries is covered with a black mold that occurs particularly on grapevines growing in warm climates. The symptoms first become evident when lesions are observed on the skin of the berry, they are generally most evident within the grapes after veraison and increase in intensity with berry damage and ripeness (**Cabañes *et al.*, 2002; Da Rocha Rosa *et al.*, 2002; Sage *et al.*, 2002; Serra *et al.*, 2003; Bellí *et al.*, 2006; Bau *et al.*, 2005; Gomez *et al.*, 2006; Guzev *et al.*, 2006; Leong *et al.*, 2006; Somma *et al.*, 2012**). In the study the genus *Aspergillus* was not detected in any stages of the grapes growth. The genus *Penicillium* seems to be more frequent in temperate and cold climates, such as those in northern Europe, whereas *Aspergillus* is more frequently associated with warmer and wetter regions (**Pitt and Hocking, 1997; Battilani *et al.*, 2006; Bejaoui *et al.*, 2006; Serra *et al.*, 2006**).

Table 2 Fungi identified in blue grape variety Frankovka modrá from July 2014 to September 2014 by the direct plating method

Fungi	Number of colonized berries							
	Pea berry	RD (%)	Early veraison	RD (%)	Harvest	RD (%)	Total	RD (%)
Alternaria	75	41.4	80	36.4	17	10.6	172	30.6
Arthrinium	0	-	0	-	1	< 1	1	< 1
Botrytis cinerea	15	8.3	17	7.7	4	2.5	36	6.4
Cladosporium	50	27.6	53	24.1	17	10.6	120	21.3
Epicoccum	7	3.9	28	12.7	25	15.5	60	10.7
Fusarium	9	5.0	5	2.3	3	1.9	17	3.0
Geotrichum	0	-	0	-	1	< 1	1	< 1
Mucor	0	-	0	-	1	< 1	1	< 1
Penicillium	12	6.6	23	10.4	79	49.1	114	20.3
Rhizopus	0	-	0	-	1	< 1	1	< 1
Trichoderma	4	2.2	6	2.7	4	2.5	14	2.5
Mycelia sterilia	9	5.0	8	3.6	8	5.0	25	4.4
Total identified fungi	181		220		161		562	

Legend: RD – Relative density

Penicillium represents 20.3% from microfungi in blue grape variety Frankovka modrá in the year 2014. The spectrum of isolated penicillia consisted of 8 species (Table 3). The most presented species of all isolates (114) was *P. expansum* (73). From this point of view, the relative density was the highest (64%).

Four species were detected at pea berry, namely *P. aurantiogriseum, P. brevicompactum, P. expansum* and *P. glabrum.* The most presented species of all isolates (12) was *P. expansum* (58.3%).

Six species were present in the veraison time, namely *P. aurantiogriseum, P. brevicompactum, P. corylophilum, P. expansum, P. glabrum* and *P. polonicum*. The largest number of isolates belonged to *P. expansum* (10, 43.5% RD), *P. brevicompactum* (5, 21.7% RD) and *P. corylophilum* (3, 13.0% RD). Another isolates were present under 10%.

The genera *Penicillium* was predominant in harvest time (49.1%), and it was represented by *P. aurantiogriseum, P. brevicompactum, P. corylophilum, P. expansum, P. glabrum, P. griseofulvum* and *P. chrysogenum*. During the harvest time, a number of *Penicillium* strains belonging to species with ability to produce mycotoxins (*P. expansum, P. griseofulvum* and *P. chrysogenum*). They were present with a 73.5% relative density of 79 *Penicillium* strains. *Penicillium verrucosum* and *P. nordicum*, the only confirmed *Penicillium* species that are able to produce ochratoxin A, were not isolated.

Fifty-nine different species of *Penicillium* have been isolated from grapes in vineyards around the world (**Rousseaux *et al.*, 2014**). The predominant species of *Penicillium* isolated from grapes differs between vineyards and vintages. For example, *P. chrysogenum* is the species most frequently isolated in Argentina (**Magnoli *et al.*, 2003**). *Penicillium brevicompactum* has been identified as the *Penicillium* species most frequently isolated from French and Portuguese vineyards (**Sage *et al.*, 2002, 2004; Serra *et al.*, 2005, 2006**). **Diguta *et al.* (2011)** identified *P. spinolusum* as the most frequently isolated species of *Penicillium*, followed by *P. expansum* and *P. minioluteum*, for the 2008 vintage, in Burgundy. **La Guerche *et al.* (2004)** identified *P. expansum* as the predominant species isolated from Bordeaux vineyards. Thus, the distribution of *Penicillium* species, which may generate organoleptic defects, depends on both vineyard and vintage. It is therefore likely to be difficult to generalize the management of filamentous fungus control (**Rousseaux *et al.*, 2014**).

Table 3 *Penicillium* species identified in blue grape variety Frankovka modrá from July 2014 to September 2014

Penicillium species	Number of colonized berries							
	Pea berry	RD (%)	Early veraison	RD (%)	Harvest	RD (%)	Total	RD (%)
P. aurantiogriseum	2	16.7	1	4.3	3	3.8	6	5.3
P. brevicompactum	2	16.7	5	21.7	7	8.9	14	12.3
P. corylophilum	0	-	3	13.0	7	8.9	10	8.8
P. expansum	7	58.3	10	43.5	56	70.9	73	64.0
P. glabrum	1	8.3	2	8.7	4	5.1	7	6.1
P. griseofulvum	0	-	0	-	1	1.3	1	<1
P. chrysogenum	0	-	0	-	1	1.3	1	<1
P. polonicum	0	-	2	8.7	0	-	2	1.7
Total	12		23		79		114	

Legend: RD – Relative density

In total 14 *Penicillium* strains representing 3 potentially toxigenic species were tested for their toxigenic ability, namely *P. expansum, P. griseofulvum* and *P. chrysogenum* (Table 4). Of all potentially toxigenic strains *P. expansum* from white grape variety Devín and *P. chrysogenum* from blue grape variety

Frankovka modrá were positive on tested mycotoxins. A 50% of *P. expansum* from Frankovka modrá were positive for citrinin, a higher percentage (80%) of isolates were positive for patulin and all were positive for roquefortin C. A small percentage (20%) of *P. expansum* isolates were positive for patulin on YES agar, whereas they were all positive for citrinin, reported **Abrunhosa *et al.* (2001)** from the wine-producing regions of Portugal. *Penicillium griseofulvum* from Frankovka modrá was positive for griseofulvin, cyclopiazonic acid and roquefortin C but not for patulin. Out of 14 strains, 64% produced et least one mycotoxin as revealed by the method used here. Patulin, produced primarily by *P. expansum* is a thermal resistant, causes gastrointestinal problems, skin rashes, and is known to be mutagenic, immunologic, and neurotoxic mycotoxin (**Abrunhosa *et al.*, 2001**). Patulin contaminates apples and apple derivates (**Frisvad and Flitenborg, 1989**). However, patulin has also been reported in grapes (**Moake *et al.*, 2005**), processed grape juice and fermenting wine (**Majerus *et al.*, 2008**). Patulin inhibits the fermenting yeast *Saccharomyces cerevisiae* in the must. It is partially degraded by the addition of sulfur dioxide and completely degraded during alcoholic fermentation (**Ough and Corison, 1980; Díaz *et al.*, 2011**). It is therefore unlikely to be present in wine. Citrinin is a nephrotoxic mycotoxin produced by several species of the genera *Aspergillus*, *Penicillium* and *Monascus*. *Penicillium citrinum*, the main producer of citrinin, was not isolat from our grapes. Citrinin is not degraded during alcoholic fermentation and may be present in very small amounts in wine. However, wine contamination is unlikely, due to the low abundance of citrinin producing species on grapes.

Table 4 Toxinogenity of selected *Penicillium* strains isolated from grapes

Penicillium species	Isolated from	C	P	G	CPA	RC
P. expansum	Devín	2*/2**	2/2			2/2
P. expansum	Frankovka modrá	5/10	8/10			10/10
P. griseofulvum	Frankovka modrá		0/1	1/1	1/1	1/1
P. chrysogenum	Frankovka modrá					1/1

Legend: C – citrinin; P – patulin; G – griseofulvin; CPA – cyclopiazonic acid; RC – roquefortin C; * positive isolates; ** number of tested isolates

CONCLUSION

Two grape varieties Devín and Frankovka modrá (Blue Frankish) were collected in the maturation stages corresponding to pea berry, early veraison and harvest from the Nitra wine region. Samples were mycological analysed with focus on genera *Penicillium*. The mycobiota changed with maturation stage. The most presented genera by descending order at pea berry and early veraison were *Alternaria, Cladosporium* and *Epicoccum* and at ripe berry *Alternaria, Cladosporium* and *Penicillium expansum*. *Penicillium* contamination in all stages types of grapes Devín was lower than in Frankovka modrá grape variety, where 79 isolates of 7 *Penicillium* species were found. Moulds commonly isolated from grape variety Frankovka modrá were *Alternaria*, *Cladosporium* and *Penicillium*. Results indicate that *Penicillium expansum* is the major species of *Penicillium* found on grapevins that can potentionally contaminate grapes with patulin. However, the average relative density of patulin-producing strains of *P. expansum* in grapes variety Devín was relatively low (1.2%) but in blue grapes higher (20.3%). Three potentially toxigenic species isolated from exogenous mycobiota were tested for their toxigenic ability by thin layer chromatography method, namely *P. expansum, P. griseofulvum* and *P. chrysogenum*. Out of 14 strains, 64% produced at least one mycotoxin. In the research, ochratoxigenic microfungi were not found in grape samples. The use of good quality raw materials is essential for mycotoxin control in food products.

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