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DIVERSITY OF BACTERIA DURING FERMENTATION OF LIMABEAN INTO *DADDAWA*

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ABSTRACT

The diversity and succession of bacteria during the natural fermentation of lima bean (*Phaseolus lunatus*) to produce *daddawa* (a fermented condiment) was studied using molecular method (16SrRNA gene analysis) with a view to develop a framework for production of *daddawa* of consistence quality with starter culture of *Bacillus* species. Lima bean was fermented for 72 h, during which isolation of bacteria and extraction of DNA were carried out. The extracted DNA of the bacterial isolates was tested for quality using agarose gel electrophoresis. The results of the 16SrRNA gene analysis were matched with the existing similar sequences in data base. Twenty six (26) presumptive isolates of *Bacillus* obtained at 24 h interval during the natural fermentation process were identified. The result of the ratio of absorbances of the extracted DNA at 260 and 280 nm showed that 73% of the isolates had pure DNA while the result of the gel electrophoresis showed well defined bands of the amplicons for the isolates. The BLAST result identified the isolates as *Bacillus amyloliquefaciens, B. subtilis, B. pumilus, B. cereus* and *B. anthrasis* with *B. subtilis* been the most predominant. *Bacillus amyloliquefaciens, B. subtilis* and *B. pumilus* occurred through-out the fermentation process. The study established the identity of the important *Bacillus* species involved in fermentation of lima bean into *daddawa* using molecular technique. These major *Bacillus* species could further be tested and developed as potential starters for improved production of *daddawa* from lima bean.

Keywords: Limabean, *daddawa*, Fermentation, *Bacillus*, 16SrRNA gene analysis

INTRODUCTION

Daddawa also known as *iru,* among the Yorubas in South-west Nigeria, is a popular condiment used as taste and flavour enhancer in soup and dishes in Africa. *Daddawa* is traditionally produced from locust beans (*Parkia biglobossa*) seeds. The tree is a leguminous plant found in the Savannah region of Africa, South East Asia and South America (**Egwim *et. al.*, 2013**). The tree is a perennial plant with pods ranging from pink, brown to dark brown, when matured. Nutritionally, African locust bean is an outstanding source of plant protein (**Elemo *et al.*, 2011**). However, locust bean trees are going into extinction from their natural habitat because they are not cultivated. Lima bean (*Phaseolus lunatus*) is an under-utilized legumes, whose seeds are good source of protein, dietary fibre, potassium, iron, copper, phosphorous, magnesium and thiamin (**WHFoods, 2010**). Previous study has shown that Lima bean could be used in production of *daddawa* (**Farinde *et al.*, 2011**).
Biochemical method has been the conventional method for identification of isolates in fermentation process to produce *daddawa* (**Abiose *et al.*, 1986; Barber *et al.*, 1988; Achi, 1992; Barimalaa *et al.*, 1994; Omafuvbe *et al.*, 2000, 2002; Farinde *et al.*, 2011**). This method may not account for minor microbial populations, stressed or injured cells that may be present in low number (**Fleet, 1999**). The biochemical method may not give a complete representation of food microbial community (**Kesmen *et al.*, 2012**). In the last decades, interests in microbial ecology have increased due to advances in molecular biology such as advent of polymerase chain reaction and DNA sequencing (**Cocolin and Ercolini, 2009**). Succession of microorganisms involved in fermentation of African locust bean seeds using culture dependent techniques have been studied by various researchers (**Abiose *et al.*,1986; Achi, 1992; Omafuvbe *et al.*, 2004; Adelekan and Nwadiuto,2012; Adewunmi *et al.*, 2013**) and it has been shown that the major microorganisms involved in the fermentation process are the *Bacillus* species. Molecular genotyping techniques are considered to be effective and rapid tool for identification and characterization of *Bacillus* (**Morten *et al.*, 2000; Miambi *et al.*, 2003**).
These techniques include restriction fragment length polymorphism (RFLP) for the grouping and typing of the isolates at species level (**Joung and Cote, 2002**), 16SrDNA sequencing for the description of phylogenetic relationships (**Ash *et al.*, 1992**) and pulse field gel electrophoresis (PFGE) for the differentiation of isolates at strain level (**Liu and Chen, 1997; Mendo *et al.*, 2000**). Identification and succession of microorganisms involved in fermentation of Lima bean to produce *daddawa* using molecular method is yet to be documented. The present study therefore employed molecular method to identify the predominant bacteria during natural fermentation of Lima bean to produce *daddawa*.

MATERIALS AND METHODS

Matured dried lima bean seeds were purchased at Ita-ogbolu, Ondo State, Nigeria. Calabashes, cooking pots, washing bowls and sample dishes were obtained from a local market in Ibadan, Nigeria. The media used were obtained from Oxoid (UK) and LAB M (UK). Primers and reagents for molecular analyses were obtained from Inqaba Biotechnology, South Africa.

Lima bean fermentation

Lima bean fermentation was carried out according to the method used by **Farinde *et al.* (2014)**. Lima bean seeds were roasted, dehulled and cooked for 40 min. The cooked beans were drained and poured while still warm into clean calabash lined with clean banana leaves, covered with banana leaves before covering with another calabash. One calabash was prepared for each fermentation stage. The calabashes with their content were placed in an incubator for fermentation to take place at 35 °C ± 2 °C for 72 h. Sampling of the Lima beans under fermentation was carried out at 24 h intervals in triplicates.

Microbial analysis

Total viable count (TVC) was determined using the method described by **Abiose *et al.* (1986)** and **Omafuvbe *et al.* (2000)**. Aliquot (1.0 ml) of appropriately diluted sample was plated in triplicates on nutrient agar (NA) plates. The plates were then incubated aerobically at 35 °C ± 2 °C for 24 h. Colonies were counted and expressed as colony forming unit per gram (cfu g^{-1}) of the sample. Representative colonies were streaked repeatedly to obtain pure isolates. Preliminary identification of the bacterial isolates was based on cultural characteristics, Gram staining reactions (**Harrigan and McCance, 1976; Harrigan, 1998**).

Molecular identification of Bacterial isolate

The isolated bacteria were identified using the method described by **Adelekan and Nwadiuto (2012)**. The experiments were carried out at National Center for Genetic Resources and Biotechnology (NACGRAB) and International Institute of Tropical Agriculture (IITA), both in Ibadan, Nigeria. The method involves DNA extraction, PCR amplification and DNA sequencing.

DNA extraction and quality verification

DNA was extracted using DNA extraction kit. ZR Fungal/Bacterial DNA MiniPrep™ protocol (ZYMO Research) following manufacturer's instructions. Modified method of **Adelekan and Nwadiuto (2012)** was used. Lysing of bacterial cells to bring out the DNA was done using bashing beads and lysis solution supplied in the DNA extraction kit instead of the method of freezing-thawing cycles used by Adelekan and Nwadiuto (2012). The extracted DNA was verified for purity and quality using quantitative and qualitative methods. Quantitative method was carried out using Nano drop spectrophotometer. DNA sample (1µl) was loaded using micro pipette into the Nano drop spectrophotometer and the absorbances of the samples were read at 260 and 280 nanometer. The concentration of the extracted DNA and the ratio of the absorbance were automatically generated and displayed on the equipment. The concentration must not be less than 10 ng/µl **(Leninger, 1975; Biobank, 2004)**. The ratio of the absorbance is also important during Polymerase chain reaction (PCR). It must be between 1.8 and 2.2 for optimum DNA concentration of high purity **(Leninger, 1975; Brown, 1993; Biobank, 2004)**.
Qualitative method was carried out using agarose gel electrophoresis. Agarose (1%) was prepared in Trisboric EDTA buffer (TBE) and melted in a microwave and cooled to 50 °C. The molten agarose was stained with Gel Green (20 µl). Gel Green fluoresce under UV light. Stained agarose was poured into gel caster in which comb had been placed at one end. The gel was allowed to solidify. The solid gel was placed in electrophoresis tank containing TBE buffer and the comb was gently removed. DNA (1 µl) was mixed with loading dye (7 µl) and loaded into wells created by the comb. The electrophoresis was allowed to run at 130 volts for about 1hour 30 min during which DNA molecules migrated through the agarose gel in the buffer.

Photographing the gel

The gel was removed from the electrophoresis tank and placed under U.V light in a documentation unit and viewed using a protective eye glasses. A Polaroid camera was placed over the documentation box and the gel was photographed. Sharp bands indicated quality DNA.

Amplification of DNA by polymerase chain reaction

Specific region of 16SrRNA gene of isolated DNA was amplified by reacting the cell solution (template DNA) (1µl) with polymerase chain reaction (PCR) master mix (8 µl), primers (1 µl) and nuclease free water (6 µl) and running the reaction cycles in a PCR thermocycler machine (Lexus Gradient –Eppendorff AG). The primers used in this study were universal primers, synthesized commercially by Inqaba Biotechnology, South Africa. The pair of primer consists of forward primer F 27 having nucleotide sequence AGAGTTTGATC(A/C)TGGCTCAG and reverse primer R 1492 having nucleotide sequence TACGG(C/T)TACCTTGTTACGACTT. Control tube (PCR reaction mixture minus template DNA) was also set along with the reaction tubes. The reaction was allowed to run 35 cycles at conditions of 94 °C for 3 minutes (Initial Denaturation), 94 °C for 1 minute (Final Denaturation), 55 °C for 30 seconds (Annealing), 72 °C for 2 minute (Extension) and 72 °C for 4 minutes (Final extension).
All PCR products were verified using 1% agarose gel electrophoresis as previously described. The samples were loaded along with Themo- scientific Gene ruler/ ladder (1 kbp).

DNA sequencing and Identification of isolates

Big Dye Terminator Cycle sequencing kit protocol was used for sequencing the amplicons and the gel was run on 3130 X 1.16 capillaries genetic analyzer from Applied Biosystems. The resultant sequences were submitted to Data base (Genbank, Germany) of National center for biotechnology Information (NCBI), the sequences were given accession numbers. The sequences were then matched with existing data in Data base using the Basic local alignment search tool (BLAST) algorithm. BLAST allows alignment of search sequence to thousands of different sequences in the data base **(Altschul *et al.*, 1997)**. Species which had less than 90 % sequence identity with known representative sequence in data base were classified as unknown species and sequences with more than 90 % sequence identity were classified as known species.

RESULTS AND DISCUSSION

Microbial Counts during Natural Fermentation of Lima Bean to Produce *Daddawa*

The result of the Total viable count of microorganisms during natural fermentation of Lima bean to produce *daddawa* is shown in Figure 1.Total viable count increased from 4.65 log cfu g^{-1} at 0 h to 7.15 log cfu g^{-1} at 24 h, reached its peak (logarithmic phase) at 48 h of fermentation (8.08 log cfu g^{-1}) after which the count dropped to 7.40 log cfu g^{-1} at 72 h of fermentation. Similar trend of an initial increase in total viable bacteria count between 0 and 48 h of fermentation and subsequent drop in the count at 72 h of fermentation during production of *daddawa* have been reported (**Abiose *et al.*, 1986; Omafuvbe *et al.*, 2000; Omafuvbe *et al.*, 2002; Enujuigba *et al.* 2008; Fadahunsi and Olubunmi, 2010**). Increase in the total count at initial fermentation phase is probably due to availability of nutrients in form of carbon and nitrogen sources.

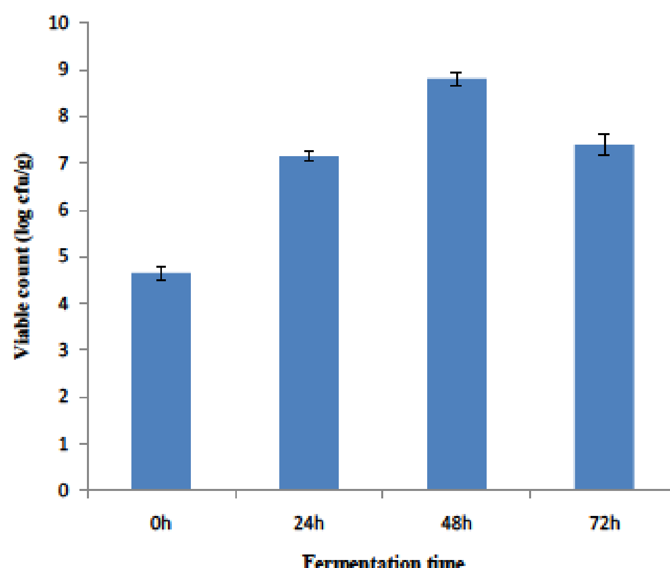

Figure 1 Total Viable Count of Microorganisms during Natural Fermentation of lima bean

Lima bean has been reported to have these nutrients in good quantities **(Yellavila *et al.*, 2015)**. The drop in TVC after 48 h was probably due to depletion of nutrients and accumulation of metabolites. Among the likely metabolites of protein hydrolysis are short peptides and ammoniacal compounds which gave the product its desired flavor. These metabolites were likely more than the metabolites produced from hydrolysis of fat and carbohydrates present in the lima beans. The possible effect of these metabolites is the alkaline pH values which the microorganisms might possibly be able to tolerate up to 48 h of fermentation, after which some of the microorganisms may find the increased alkaline environment unfavorable for their growth and proliferation and so the total number of microorganisms declined.

Preliminary identification of bacterial isolates

Bacteria isolated from natural fermentation of lima bean to produce *daddawa* are shown in Table 1. The isolates were Gram positive, rods, catalase positive and spore formers. These characteristics allowed preliminary identification of *Bacillus* **(Harrigan and MacCance, 1976; Harrigan, 1998)**. Bacterial isolates 3, 9, 19 and 23 had rhizoid shape, isolates 7,13,16,18 and 20 had circular shape while the rest of the bacterial isolates (1, 2, 4, 5, 6, 8, 10, 11, 12, 14, 15, 17, 21, 22,24, 25 and 26) had irregular shape on agar plate. Similar preliminary phenotypic identification of bacterial isolates from *iru, afitirin* and *sonru* as belonging to genus *Bacillus* was reported by **Azokpota *et al.* (2007)**. This preliminary identification which showed the bacterial isolates as *Bacillus* was used to search for the appropriate primers for the molecular identification of bacteria isolates.

Table 1 Preliminary identification of Bacterial Isolates from Natural Fermentation of Lima Bean to Produce *Daddawa*

Isolate number	Appearance on agar plate	Shape	Gram's reaction	Catalase reaction	Presence of spore
1	Irregular	Rod	+ve	+ve	+ ve
2	Irregular	Rod	+ve	+ve	+ ve
3	Rhizoid	Rod	+ve	+ve	+ve
4	Irregular	Rod	+ve	+ve	+ ve
5	Irregular	Rod	+ve	+ve	+ ve
6	Irregular	Rod	+ve	+ve	+ ve
7	circular	Rod	+ve	+ve	+ ve
8	Irregular	Rod	+ve	+ve	+ ve
9	Rhizoid	Rod	+ve	+ve	+ ve
10	Irregular	Rod	+ve	+ve	+ ve
11	Irregular	Rod	+ve	+ve	+ ve
12	Irregular	Rod	+ve	+ve	+ ve
13	Circular	Rod	+ve	+ve	+ ve
14	Irregular	Rod	+ve	+ve	+ ve
15	Circular	Rod	+ve	+ve	+ ve
16	Circular	Rod	+ve	+ve	+ ve
17	Irregular	Rod	+ve	+ve	+ ve
18	Circular	Rod	+ve	+ve	+ve
19	Rhizoid	Rod	+ve	+ve	+ ve
20	Circular	Rod	+ve	+ve	+ ve
21	Irregular	Rod	+ve	+ve	+ ve
22	Irregular	Rod	+ve	+ve	+ ve
23	Rhizoid	Rod	+ve	+ve	+ ve
24	Circular	Rod	+ve	+ve	+ ve
25	Irregular	Rod	+ve	+ve	+ ve
26	Irregular	Rod	+ve	+ve	+ ve

Sequencing and BLAST result

Forward primer 27F and reverse primer 1492R were used to amplify the 16SrRNA gene of each of the bacterial isolates which resulted in well defined amplicons. When the amplicons were sequenced, the base sequences ranged from 911 in isolate number 8 to 1199 base sequence in isolate number 9 (Table 2).
When the sequences were matched with already existing similar sequences in Gene Bank Data base, all the isolates have their identity between 94 and 98% as shown in Table 2. Spore forming *Bacillus* species were identified as the major bacteria present during the fermentation process. Four isolates (isolates 12, 13, 15 and 24) did not match any identity. The identified isolates fell into 5 species of *Bacillus* (Table 3).These include *Bacillus amyloliquefaciens, Bacillus subtilis, Bacillus pumilus, Bacillus cereus and Bacillus anthracis*.
From the BLAST result, *Bacillus subtilis* was identified as the most predominant species during the fermentation of lima bean to produce *daddawa* (Table 3). **Azokpota *et al.*** (2007), who used ITS – PCR – RFLP analysis to identify the bacteria isolated from three *daddawa* products (*iru, afitirin* and *sonru*), reported that *Bacillus subtilis* group represented the dominant species in the three condiments (*iru, afitirin* and *sonru*). **Adewunmi *et al.* (2013)** also reported that the result of polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE) identified *Bacillus subtilis* as the consistent bacterial species associated with fermentation of *iru*. Previous studies by several authors using culture dependent methods and biochemical characterization have also reported *Bacillus subtilis* as being predominant during fermentation of beans to produce

Table 2 Result of the 16SrRNA Squence Analysis of the Bacterial Isolate

Isolate	Identity	(%) Similarity	Base sequence	Accession Number
1	*B. amyloliquefaciens* Strain FGYM6	98	1173	JN999853.1
2	*B. subtilis* Strain ov	98	1196	GU585579.1
3	*Bacillus sp.* Strain OS 1	98	1181	EF428970.1
4	*B. anthracis* Strain R5-331	97	1120	JQ65973.1
5	*B. anthracis* Strain yxc1-1	97	1050	JF701962.1
6	*B. subtilis* Strain C3	95	1179	JX120508.1
7	*B. cereus* Strain 25	95	1089	DQ42176.1
8	*B. subtilis* Strain 30L1-2	94	911	JN366795.1
9	*Bacillus sp.* Strain EGY-WCP9	95	1199	KF5623361.1
10	*B. subtilis* Strain BG-B7	97	1164	EU869248
11	*B. anthracis* Strain R5-331	97	1193	JQ659732.1
14	*B. pumilus* Strain DL-006	98	1151	KJ608548.1
16	*B. cereus* Strain VIT-AVJ	97	1174	KJ437489.1
17	*B. amyloliqueficiens*	97	1147	KC492052
18	B. *cereus* Strain HC23	97	1175	KJ206081.1
19	*Bacillus sp.* Strain HB38	96	1191	KF8638337.1
20	*B. cereus* Strain 400	98	1145	DQ420187.1
21	*B. amyloliquefaciens* Strain D.18	98	1146	AB813716.1
22	*B. amyloliquefaciens* Strain NK3-1	98	1158	HQ831391.1
23	*Bacillus. sp.* Strain HB38	97	1171	KF863837.1
25	*B. anthracis* Strain R5331	94	1154	JQ659732.1
26	*B. subtilis* Strain AB30	97	1142	JX188065.1

B - Bacillus

condiments (**Antai and Ibrahim, 1986; Odunfa and Oyewole, 1986; N'dir *et al.*, 1994; Omafuvbe *et al.*, 2000; Omafuvbe *et al.*, 2002; Ouoba *et al.*, 2004; Okpara *et al.*, 2013**). *Bacillus species* have been reported to be associated with fermentation of vegetable proteins (**Abiose *et al.*, 1986: Kiers *et al.*, 2000; Omafuvbe *et al.*, 2002; Enujiugha, 2009; Ojinaka and Ojimelukwe, 2013**). Inability to identify 15 % of the total isolates could probably be linked with some inadequacies in the nucleotide sequence in the genes of the representative chromosomes with which the primers could bind. **Azokpota *et al.* (2007)** also

reported that 16 % of isolates from *iru* and *sonru* (*daddawa*-like) could not be identified with molecular method.
Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus pumilus were found to occur throughout the fermentation period (Table 4). The occurrence of *Bacillus subtilis* throughout the fermentation period and its predominance in fermenting beans has been reported by various authors (**Ogueke and Ariatu, 2004; Achi, 2005; Omafuvbe, 2008; Adelekan and Nwadiuto, 2012**). *Bacillus subtilis* was identified as the best starter culture for fermentation of soybean to produce *daddawa* (**Omafuvbe *et al.*, 2002**). Of particular interest is *Bacillus amyloliquefacens* which also occur

Table 3 *Species* of *Bacillus* identified by 16SrRNA Sequence Analysis Using Basic Local Alignment Search Tool (BLAST)

Isolate number	% similarity	Bacterial *specie*
1, 17, 21, 22	98, 98, 98 and 98 respectively	*Bacillus amyloliquefaciens*
2, 6, 8, 10, 26	98, 94, 94, 97 and 97 respectively	*Bacillus subtilis*
3, 9, 19 and 23	98, 95, 96 and 97 respectively	*Bacillus species*
14	98	*Bacillus pumilus*
7, 16, 18, 20	95, 97, 97 and 98 respectively	*Bacillus cereus*
4, 5, 11, 25	97, 97, 97 and 94 respectively	*Bacillus anthracis*
12,13,15 and 24	No matched identity	

Table 4 Bacterial Succession during Natural Fermentation of Lima Bean to Produce *Daddawa*

Bacteria	Fermentation time (h)			
	0	24	48	72
Bacillus subtilis	+	+	+	+
B. amyloliquefaciens	+	+	+	+
B. pumilus	+	+	+	+
B. cereus	+	+	-	-
B. anthracis	+	+	-	-

+ = present - = not present

through-out the fermentation process. *Bacillus amyloliquefaciens* is a plant associated bacterium which is known for its ability to promote host plant growth through production of stimulating compounds and suppression of soil borne pathogens by synthesizing antibacterial and antifungal metabolites (**Niazi *et al.*, 2014**). Of concern is the occurrence *Bacillus cereus* and *Bacillus anthracis* in the fermentation process as their presence in the fermented product could pose public health problems (**Adelekan and Nwadiuto, 2012**). Occurrence of these organisms did not exceed 24 h of fermentation, an indication that they are not important in the fermentation of lima bean. **Adelekan and Nwadiuto (2012)** similarly reported occurrence of *Bacillus cereus* and *Bacillus anthracis* during the first 48 h of fermentation of locust bean to produce *daddawa*. *Bacillus cereus* and *Bacillus anthracis* have been reported to be members of cultivable bacteria associated with fermented beans (**Choma and Granum, 2002; Ouoba *et al.*, 2004; Oguntoyinbo *et al.*, 2010**).
The occurrence of *Bacillus cereus* and *Bacillus anthracis* or any other pathogen in the early stages of fermentation process could probably have arisen from handling of the beans after boiling and their inability to occur at the later stages of fermentation might be due to unfavorable environment for their survival (**Omafuvbe *et al.*, 1999**) coupled with the of presence of *B. amyloliquefaciens* which might probably suppress or inhibit growth of pathogens. However, food borne diseases have not been reported in areas where these condiments (*iru/daddawa, afitirin, soniru)* are generally consumed (**Azokpota *et al.*, 2007**). **Azokpota *et al.* (2007)** thus suggested that further medical investigation have to be carried out to confirm whether *Bacillus cereus* strains identified in fermented condiments such as *iru/daddawa* are actually toxigenic.
There were no fungi isolated in the Lima bean fermentation process. This might be as a result of heat process prior to fermentation and probably an uncondusive environment for their growth and multiplication. Absence of fungi in the fermented Lima beans makes the product safe for consumption in terms of mycotoxin production (***Abiose et al.*, 1986**).

CONCLUSION

The study concluded that molecular method could be used to identify the important microorganisms in fermentation of lima bean to produce *daddawa*. The method revealed that *Bacillus subtilis, B. pumilus* and *B.amyloliquefaciens* were majorly involved in the fermentation process with *B. subtilis* been predominant and that these three species of *Bacillus* could be tested and purified for use as potential starter for producing quality lima bean *daddawa*.

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ISOLATION OF LIPOLYTIC BACTERIA FROM COLOMBIAN ANDEAN SOILS: A TARGET FOR BIOPROSPECTING

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ABSTRACT

Microbial enrichments with a substrate of interest could enhance the possibility of finding certain desired metabolic activities. As lipases are one of the most important enzymes in industrial applications, the Colombian Andean soils were explored as a source of lipolytic microorganisms. Two Andean soils under low temperatures were sampled: paramo and glacier soils from "*Los Nevados*" National Natural Park. Both soils were enriched through a fed-batch fermentation using olive oil as the inductor substrate. Forty-three lipolytic isolates were obtained and their taxonomic assignments were performed on the basis of 16S rDNA gene sequencing. In both cases, the phylum Proteobacteria represented the majority of the isolates. Qualitative assays to measure the lipolytic activity were performed by using tributyrin, triolein or olive oil (1%). Two isolates identified as *Pseudomonas psychrophila* and *Stenotrophomonas rhizophila* produced the largest hydrolysis halos, with an optimal activity at pH 8 and 50°C. Only the *S. rhizophila* extracellular fraction hydrolyzed short and long chain pNP-esters, including pNP-palmitate. The broader substrate specificity of this isolate is probably due to the simultaneous presence of lipase and esterase activity in the crude extract. This is the first report of lipolytic activity in *S. rhizophila*, and the first preliminary characterization of the novel lipolytic activity in *S. rhizophila* and *P. psychrophila*. Further work is needed to purify and completely characterize the esterases and lipases produced by both species.

Keywords: 16S rDNA, Fed-batch fermentation, lipase, psychrotrophs

INTRODUCTION

Lipases are important biocatalysts with innumerable applications in food, dairy, detergent and pharmaceutical industries. The biological function of these enzymes is to catalyze the hydrolysis of triacylglycerols to release free fatty acids, diacylglycerols, mono-acylglycerols and glycerol (**Patil *et al.*, 2011**). Lipolytic enzymes include esterases commonly defined as carboxyesterases (EC. 3.1.1.1) and lipases, originally called true lipases (**Sangeetha *et al.*, 2011**). Over the last few decades, lipases have gained special attention for their ability to act in micro-aqueous environments and catalyze esterification, trans-esterification, aminolysis and acidolysis reactions (**Joseph *et al.*, 2008**).
Bacterial lipases have valuable properties such as a wide variety of substrates, selectivity, stability in organic solvents, and activity in harsh conditions. Other advantages that promote research on these enzymes are their high yield, low production costs and the possibility of genetic manipulation (**Glogauer *et al.*, 2011; Meilleur *et al.*, 2009**). Most of the lipases in industry are lipoproteins found in genera such as: *Achromobacter, Acinetobacter, Bacillus, Burkholderia, Pseudomonas, Staphylococcus, Chromobacterium,* among others (**Castilla *et al.*, 2017; Soliman *et al.*, 2007**). These lipases can be used in different applications including production of fats and oils, detergents, fine chemicals, pharmaceutical drugs, pulp and paper, as well as use in bioremediation, waste treatment, oil biodegradation and medical applications (**Stergiou *et al.*, 2013**).
The low stability of well-characterized mesophilic enzymes under extreme conditions has prompted the search for new enzymes with better properties (**Tirawongsaroj *et al.*, 2008; Jiménez *et al.*, 2012; López *et al.*, 2014**). Thus, the isolation of novel microorganisms capable of producing stable lipases for industrial applications is required (**Messiasa *et al.*, 2009; Abol *et al.*, 2016**). Extreme environments with low temperatures are the most common places to find psychrophilic bacteria with lipolytic activity (**De Pascale *et al.*, 2012; De Santi *et al.*, 2016**). Cold-adapted microorganisms produce lipases which function effectively at cold temperatures with high rates of catalysis, in comparison to those lipases synthesized by mesophiles or thermophiles that show low, or no activity at low temperatures (**Joseph *et al.*, 2008**). Some of the adaptations found in enzymes from extreme environments include structural flexibility, higher content of alpha helix compared to beta- sheets and temperature independent reaction rates (**Dalmaso et al., 2015**).
Previous studies have reported direct functional screening to isolate lipolytic microorganisms through enrichment cultures with lipid substrates including oils (olive oil, palm oil, oil mill), fatty acids and glycerol (**Yao *et al.*, 2013; Gombert *et al.*, 1999**). Hence the enrichment cultivation in olive oil used in this study, was carried out based on the assumption that microorganisms able to survive on this substrate can synthesize lipases (**Gaoa *et al.*, 2000**). One of the advantages of this technique is the accumulation of microorganisms with useful enzymes in growth-controlled conditions (**Ekkers *et al.*, 2012**).
The major aim of this study was to isolate lipolytic bacteria from low temperature environs found in the paramo and glacier soils from "*Los Nevados*" National Natural Park. The screening of lipolytic bacteria through the enrichment culture strategy, followed by the bacterial identification by 16s rDNA sequencing, and the preliminary enzyme characterization at different conditions (temperatures, pH and substrates) were the main steps that allowed us to bioprospect for putative novel lipases.

MATERIALS AND METHODS

Soil samples

Samples were taken from two cold environments in "*Los Nevados*" National Natural Park (Manizales – Colombia). The glacier soil sample (4°47´44.98" LN 75°21´52.33" LW at 5200 MAMSL) registered 4,1°C and pH 4,18. The sample from Paramo, rhizosphere-associated (4°51´51.1" LN 75°21´13.7" LW at 4322 MAMSL) registered 9°C and pH 4,88. Both samples were taken between 10 and 15 centimeters depth. The physico-chemical soil analysis was determined according to **Tedesco *et al.*, (1995)** including: pH, soil texture, electrical conductivity E.C. (ds/m), cation exchange capacity C.E.C (me/100), organic carbon percentage (%OC), phosphorus, potassium, sulfur, calcium, iron, and magnesium. All samples were stored at 4°C until they were used.

Enrichment cultures

The inoculum preparation started with the samples being washed in mineral salt medium (MSM), supplemented with tween 20 (0.5%) and olive oil 1%. This procedure was performed with eight grams of soil for each environment, followed by a 24 hours incubation at 140 rpm and at 22°C (**Wang *et al.*, 2005**). After sedimentation, the supernatant was used as inoculum (10%) for the enrichment culture. This was performed with the same supplemented medium, in a fermenter batch adjusted to 1L bioreactor. The effective volume was 70%, and the cultures conditions were 22°C, 140 rpm, pH 6.9 and 1.6 L min^{-1} of aeration during 3 weeks (**Lanser *et al.*, 2002; Henne *et al.*, 2000**). Every 5 days the bioreactor was fed with MSM ensuring the final effective volume. CFU mL^{-1} was estimated in nutrient agar (MoBio), (**Köhler, 2007; Wang *et al.*, 2005**).

Isolation and morphological description

Functional screening was carried out at the end of the enrichment in MSM agar supplemented with olive oil or tributyrin at 22°C after three days (**Bunterngsook *et al.*, 2010; Ertugrul *et al.*, 2007**). Colonies with hydrolysis halos were isolated and preserved in 15% glycerol solution at -80°C. The morphological description of colonies was performed and Gram staining was also included to verify cell shape and cell wall properties.

Lipase assay

Lipase production and secretion was evaluated in triplicate by agar diffusion assay. Briefly, MSM agar plates were supplemented with 1% of tributyrin, olive oil or triolein (**Chakravarthy & Narasu, 2012**). As preinoculum, one full loop of the isolated colonies was transferred in 10 ml LB broth at 20°C for 24 hours. After incubation, 5 µl of the inoculum was then transferred on filter paper disks and incubated on agar plates at 20°C for 8 days. The strains were examined daily for their ability to produce clarified zones (**Golani *et al.*, 2016**).

DNA extraction, amplification and sequencing of 16S rDNA gene

Total DNA was extracted from the isolates as previously described (**Spanevello *et al.*, 2002**). Universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (**Sarkar *et al.*, 2014**) were used to obtain a PCR product of 1.5 Kb. A 100 µL reaction contained buffer 1X, MgCl 1.5 mM, dNTPs 0.2 mM, 0.3 µM of each primer, 0.5% of BSA and Taq Polymerase Promega 1U. PCR was carried out by an initial denaturation at 94°C for 5 min; then 30 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 45 sec, extension at 72°C for 1 min y 30 sec and finally an extension cycle at 72°C for 7 min. PCR products were analyzed by electrophoresis 1.5% (wt/vol) agarose gels with SyBR-safe (InvitroGen™). The PCR products were sequenced via the Sanger method by Macrogen Inc. Korea.

Sequence alignments and phylogenetic analysis

The 16S rDNA sequences were analyzed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and the best hit was assigned to the query. Distance trees were constructed using the UPGMA algorithm, (**Bisht & Panda, 2011**) with the MEGA 7.0® package. The alignments were performed through CLUSTAL omega http://www.ebi.ac.uk/Tools/msa/clustalo0w2/). Bootstrap resampling analysis for 1000 replicates was performed to estimate the confidence of tree topologies (**Felsenstein, 1985**). The sequences were submitted to the European Molecular Biology Laboratory (EMBL-ENA) Nucleotide Sequence Database (http://www.ebi.ac.uk/embl/Submission/webin.html). The accession numbers of the nucleotide sequence data are listed in Table 1.

Growth curve experiments

The three strains that produced the largest hydrolysis halos, *Stenotrophomonas rhizophila* (strain USBA 843E), *Pseudomonas psychrophila* (strains USBA 844A, USBA 846E) were inoculated in Bioscreen® plates in triplicate with 200 µl as final volume and an OD_{540} adjusted to 0.02. Optical density was determined at 20°C each 6 h for 80 hours in order to obtain the their growth curves.

Lipase activity

USBA 843E, USBA 844A and USBA 846E strains were cultured in LB broth supplemented with olive oil 1% as inductor substrate at 20°C for 72 hours and 140 rpm. Then, they were centrifuged at 10.000 rpm for 7 minutes to recover the supernatant. The protein content was determined using the Bradford method (**Bradford, 1976**) and the enzymatic reaction was measured in triplicate in the Bioscreen®. For this reaction, 20 µl of supernatant were added to 180 µl of buffer Tris-HCl 0.1 M and 0.1 mM of lipid substrate emulsified in isopropanol (**Rashid *et al.*, 2001**). The pNP-esters evaluated were: acetate, butyrate, decanoate and palmytate at 5°C, 10°C, 20°C, 30°C, 40°C, 50°C and 60°C. The optimum pH of lipolytic activity of the strains was determined between 3 and 9, using 0.1M Glycine-HCl pH 3; 0.1M Acetate pH 4 and 5; 0.1 M phosphate pH 6; 0.1M Tris-HCl pH 7.8 and 9. This assay was carried out for 1 h at 30°C including biological triplicates. The activity was evaluated measuring the liberated p-nitrophenol at 405 nm. One unit of activity was defined as the amount of enzyme needed to release 1 µmol of p-nitrophenol per min.

Statistical analyses

The results obtained from the lipase activity assay were analyzed through the SPSS software (version 12.0). The analyses were performed using the Shapiro-Wilk Normality Test, and the pairwise comparison Kruskal-Wallis (Non-parametric) test to establish differences between the three substrates. The homogeneous subgroups test was used to select the strains with the best lipolytic activity.

RESULTS

Physico-chemical soil properties

The PNN glacier soil had special physicochemical properties due to its lack of vegetation, low temperature (4.1°), acidity (4.18), loamy texture, low percentage of organic Carbon (0,23%), high concentration of iron (385 ppm), phosphorus (94 ppm) and sulfate (88 ppm). Moreover, Paramo soil showed a similar pH (4.88), but a silt loam texture, with higher concentrations of potassium (85 ppm), calcium (333 ppm) magnesium (58 ppm), and organic carbon (5,7%), probably influenced by the presence of vegetation like *Espeletia* and *Calamagrostis*.

Enrichment culture and isolation of lipolytic bacteria

The initial pH was adjusted to 7 ± 0.2, and it decreased around 6.0 after 3 days. Regarding cell density, the paramo enrichment culture increased its initial cell count from10^5 CFU mL^{-1} to 10^9 CFU mL^{-1}, while the glacier enrichment increased from 10^3 CFU mL^{-1} to 10^8 CFU mL^{-1}. After the screening a total of 43 colonies were isolated, 30 from the paramo enrichment and 13 from the glacier.

Morphological description

The most common colony morphologies for the bacterial isolates were: wrinkled, mucoid, viscous, and translucent, with irregular and regular shapes, and with white, pale yellow or yellow tones. Exopolysaccharide production was observed in some isolates. Some colonies could change the morphotype after transferring to a new agar plate. Nevertheless, the microscopic observation showed the same Gram-negative, rod-shaped bacteria.

Lipase assay

After 8 days of incubation at 20°C, six of the 43 isolates presented significant hydrolysis halos (statistical analysis). Isolates USBA 851A (*Pseudomonas fragi*), USBA 852C (*Ralstonia pickettii*), USBA 843E (*Stenotrophomonas rhizophila*), and USBA 844A (*Pseudomonas psychrophila*) produced the major hydrolysis halos in the tributyrin agar plates with diameters between 7 mm to 11 mm (Excluding the colony diameter). In triolein, the isolates USBA 844A and USBA 846E (*Pseudomonas psychrophila*) showed halos between 8 mm and 10 mm. In olive oil, the isolates USBA 851A (*Pseudomonas fragi*), USBA 851D (*Pseudomonas fragi*) and USBA 846E (*Pseudomonas psychrophila*) produced halos between 5 and 7 mm of diameter (Fig 1).

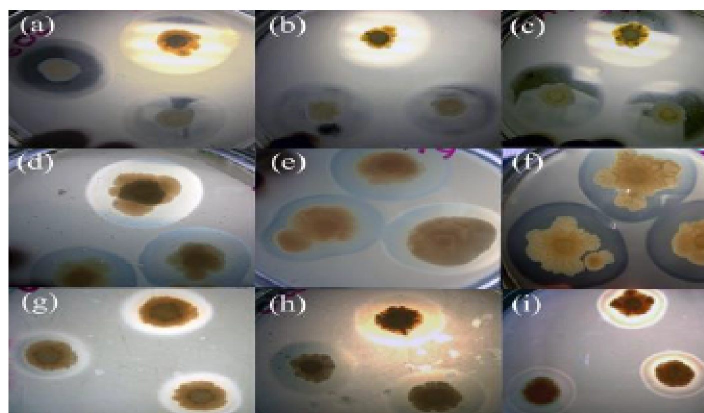

Figure 1 Hydrolysis halos obtained in tributyrin agar plates: a. USBA 851A b. USBA 852C c. USBA 844A. Triolein: d. USBA 851A e. USBA 844A f. USBA 846E. Olive oil: g. USBA 851A h. USBA 851D i. USBA 846E

As previously mentioned, *Stenotrophomonas rhizophila* and *Pseudomonas psychrophila,* among other isolates, produced the largest hydrolysis halos on the evaluated substrates. We note this fact because the lipolytic activity from *Stenotrophomonas rhizophila* has not been reported until now. This species of *Stenotrophomonas* is characterized by the absence of lipase and β-glucosidase production (Wolf et al., 2002). Additionally, the lipolytic activity of *Pseudomonas psychrophila* has been reported but it has not been evaluated. This finding led us to choose these isolates (USBA 843E, USBA 844A/USBA 846E) to perform the enzymatic activity assays on, in order to find novel lipases from psychrophilic bacteria.

Sequence alignments and phylogenetic analysis

The 16S rDNA gene sequences were compared with sequences reported in the GeneBank database using BLAST and the Ribosomal Database Project (RDPII), in order to determine the taxonomic assignment of the 43 isolates. The percent identities obtained were 99% and 100%, while the e-value was equal to zero (Tab 1).

Table 1 Taxonomic assignment of lipolytic bacterial isolates using BLASTN and accession numbers.

Code	Nearest BLAST (AC NCBI)	% Identity	Accession numbers
Glacier			
USBA 849A	*Pseudomonas mandelii* (LT629796.1)	99%	LT627086
USBA 843A	*Stenotrophomonas rhizophila* (KY474340.1)	99%	LT627087
USBA 850A	*Burkholderia fungorum* (LN868266.1)	100%	LT627088
USBA 852A	*Ralstonia pickettii* (KT354655.1)	100%	LT627089
USBA 843B	*Stenotrophomonas rhizophila* (KY474340.1)	99%	LT627090
USBA 852B	*Ralstonia pickettii* (KT354650.1)	100%	LT627091
USBA 849B	*Pseudomonas mandelii* (KU921563.1)	100%	LT627092
USBA 843C	*Stenotrophomonas rhizophila* (KR065721.1)	100%	LT627093
USBA 851A	*Pseudomonas fragi* (KX588592.1)	99%	LT627094
USBA 852C	*Ralstonia pickettii* (KY523570.1)	100%	LT627095
USBA 851B	*Pseudomonas fragi* (KX588591.1)	100%	LT627096
USBA 851C	*Pseudomonas fragi* (KX068625.1)	99%	LT627097
USBA 853A	*Dermacoccus nishinomiyaensis* (CP008889.1)	99%	LT627098
Paramo			
USBA 841A	*Aeromonas bivalvium* (KY124169.1)	99%	LT627099
USBA 842A	*Aeromonas veronii* (KY124169.1)	99%	LT627100
USBA 842B	*Aeromonas veronii* (KY124169.1)	99%	LT627101
USBA 843D	*Stenotrophomonas rhizophila* (KR065721.1)	99%	LT627102
USBA 843E	*Stenotrophomonas rhizophila (KM114951.1)*	99%	LT627103
USBA 843F	*Stenotrophomonas rhizophila (KM114951.1)*	99%	LT627104
USBA 844A	*Pseudomonas psychrophila (LT629795.1)*	100%	LT627105
USBA 843G	*Stenotrophomonas rhizophila (KR065721.1)*	99%	LT627106
USBA 843H	*Stenotrophomonas rhizophila (KY474340.1)*	99%	LT627107
USBA 849C	*Pseudomonas mandelii (LT629796.1)*	99%	LT627108
USBA 843I	*Stenotrophomonas rhizophila (KR065721.1)*	99%	LT627109
USBA 843J	*Stenotrophomonas rhizophila (KM114951.1)*	99%	LT627110
USBA 843K	*Stenotrophomonas rhizophila (KR065721.1)*	99%	LT627111
USBA 843L	*Stenotrophomonas rhizophila (KR259225.1)*	99%	LT627112
USBA 843M	*Stenotrophomonas rhizophila (KR259225.1)*	99%	LT627113
USBA 846A	*Pseudomonas psychrophila (KU173827.1)*	99%	LT627114
USBA 842C	*Aeromonas veronii (KY124169.1)*	99%	LT627115
USBA 846B	*Pseudomonas psychrophila (LT629795.1)*	100%	LT627116
USBA 845A	*Pseudomonas lini (KM114930.1)*	100%	LT627117
USBA 846C	*Pseudomonas psychrophila (LT629795.1)*	99%	LT627118
USBA 846D	*Pseudomonas psychrophila* (LT629795.1)	99%	LT627119
USBA 851D	*Pseudomonas fragi* (KX588592.1)	100%	LT627120
USBA 846E	*Pseudomonas psychrophila (LT629795.1)*	100%	LT627121
USBA 847A	*Pseudomonas jessenii (KU725946.1)*	99%	LT627122
USBA 843N	*Stenotrophomonas rhizophila (KR065721.1)*	99%	LT627123
USBA 843O	*Stenotrophomonas rhizophila (KR259225.1)*	99%	LT627124
USBA 843P	*Stenotrophomonas rhizophila (KR065721.1)*	99%	LT627125
USBA 848A	*Pseudomonas congelans (LN774224.1)*	99%	LT627126
USBA 843Q	*Stenotrophomonas rhizophila (KY474340.1)*	99%	LT627127
USBA 849D	*Pseudomonas mandelii (JX122162.1)*	99%	LT627128

The distance tree from glacier enrichment culture includes 4 clades from the *phylum* Proteobacteria, which are associated with the genera *Pseudomonas, Stenotrophomonas, Ralstonia* and *Burkholderia.* The fifth clade belongs to the *phylum* Actinobacteria, represented by the genus *Dermacoccus* (Fig 2a). The paramo enrichment culture tree showed only 3 clades. The isolates belong to the *phylum* Proteobacteria represented by the genera *Aeromonas, Pseudomonas* and *Stenotrophomonas* (Fig 2b).

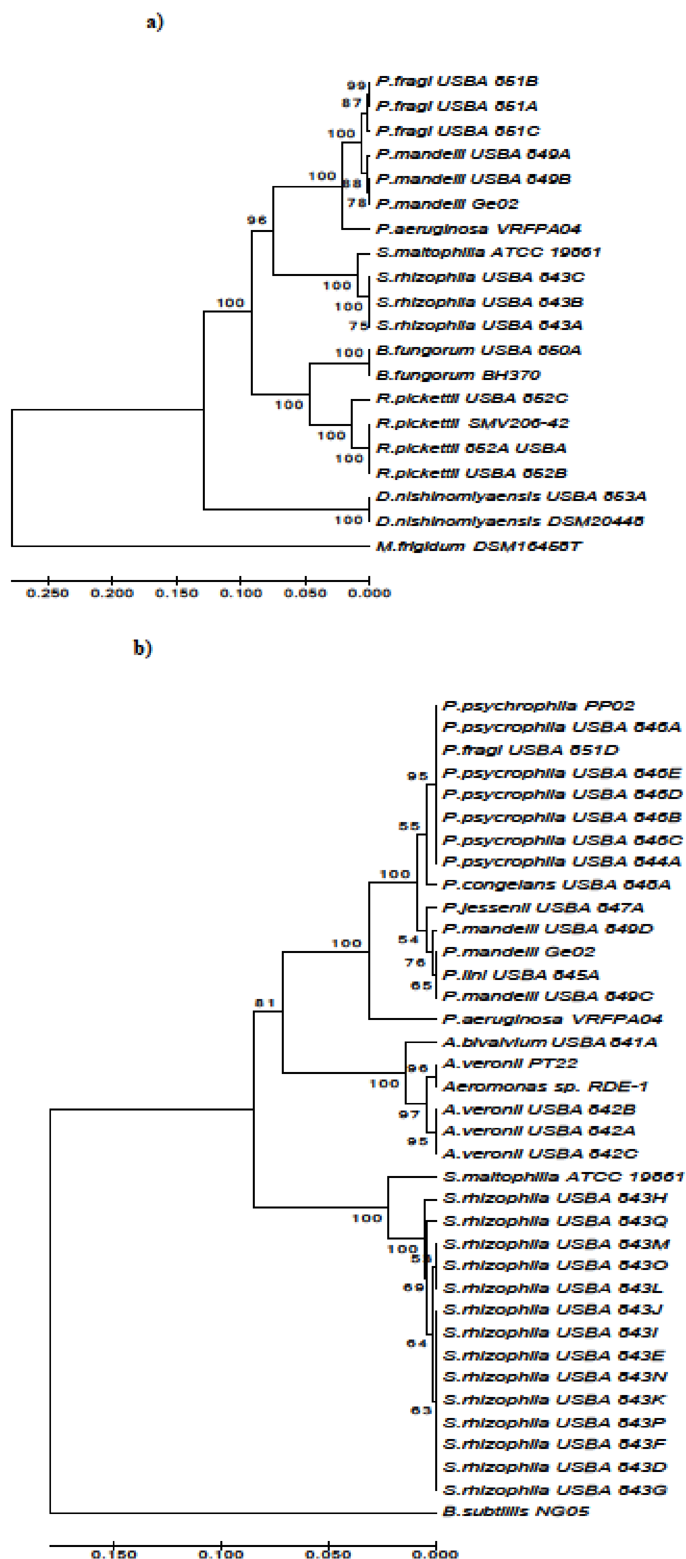

Figure 2 Phylogenetic trees generated with the 16S rDNA gene sequence from a) glacier enrichment culture b) paramo enrichment culture, compared with selected reference sequences: *P. mandelii* Ge02 (KR088369.1), *P. aeruginosa* VRFPA04 (CP008739.2), *S. maltophilia* ATCC 19861 (NR_040804.1), *B. fungorum* BH370 (LN868266.1), *R. pickettii* SMV206-42 (KT354655.1), *D. nishinomiyaensis* DSM20448 (NR_044872.1), *P. psychrophila* PP02 (KU173827.1), *A. veronii* PT22 (KY124169.1), and *Aeromonas sp.* RDE-1 (KY365513.1).The trees were generated using the UPGMA method and were rooted with *Methanogenium frigidum* (NR_104790.1) and *Bacillus subtillis* (JQ433875.1) as out groups. Scale bar indicates number of substitutions per site and the bootstrap analysis was performed on the basis of 1000 replicates

Growth curve experiments

The late stationary phase from the isolates USBA 843E (*Stenotrophomonas rhizophila*), USBA 844A and USBA 846E (*Pseudomonas psychrophila*) was reached after 72 hours of incubation. In this phase, the extracellular fraction was obtained to perform the p-nitrophenyl ester assays.

Lipase activity

The protein concentrations obtained from the extracellular fraction were: 0.108 mg mL^{-1} for the strain USBA 843E (*S. rhizophila*), 0.126 mg mL^{-1} for USBA 844A (*P. psychrophila*) and 0.092 mg mL^{-1} for USBA 846E (*P. psychrophila*). The lipase activity essay was carried out for 1 h at 30°C with four lipid substrates: pNP-acetate, pNP-butyrate, pNP-decanoate and pNP-palmitate. Short-chain pNP-esters (C2 and C4) were hydrolyzed for the three isolates. Only the USBA 843E (*S. rhizophila*) showed activity with the four substrates including pNP-palmitate. The broader substrate specificity of this isolate is probably due to the simultaneous presence of lipase and esterase activity in the crude extract (Fig 3).

Effect of temperature on enzyme activity

The USBA 843E (*S. rhizophila*) enzyme fraction showed maximal activity at 50°C in the four lipid substrates. Its highest activity was in short chain (C2 and C4) fatty acids. The extracellular fraction from USBA 844A (*P. psychrophila*) had high activity at 50°C in pNP-butyrate and at 40°C in pNP-acetate. Finally, the USBA 846E (*P. psychrophila*) showed its optimum activity at 50°C in pNP-acetate, pNP-butyrate and pNP-decanoate, and its highest activity was in short chain (C2 and C4) fatty acids (Fig 3). The pH used during the temperature assays was 7.

Effect of pH on lipase activity

The three isolates showed their optimal lipolytic activity at pH 8 in all the lipid substrates except for pNP-butyrate that was highly hydrolyzed at pH 9. For the isolate USBA 843E (*S. rhizophila*), the hydrolytic activity was observed at pH 7 in C2 and C4, pH 8 in all the lipid substrates, and pH 9 in C4 and C10 (Fig 3). The lipase from USBA 844A (*P. psychrophila*) showed activity at pH 7-8 in C2, and at pH 8-9 in C4 (Fig 3). For the isolate USBA 846E (*P. psychrophila*), the C2 hydrolysis was carried out at pH 6 to 9; of C4 at pH 8-9 and C10 at pH 8 (Fig 3). The temperature used during the pH assays was 20°C.

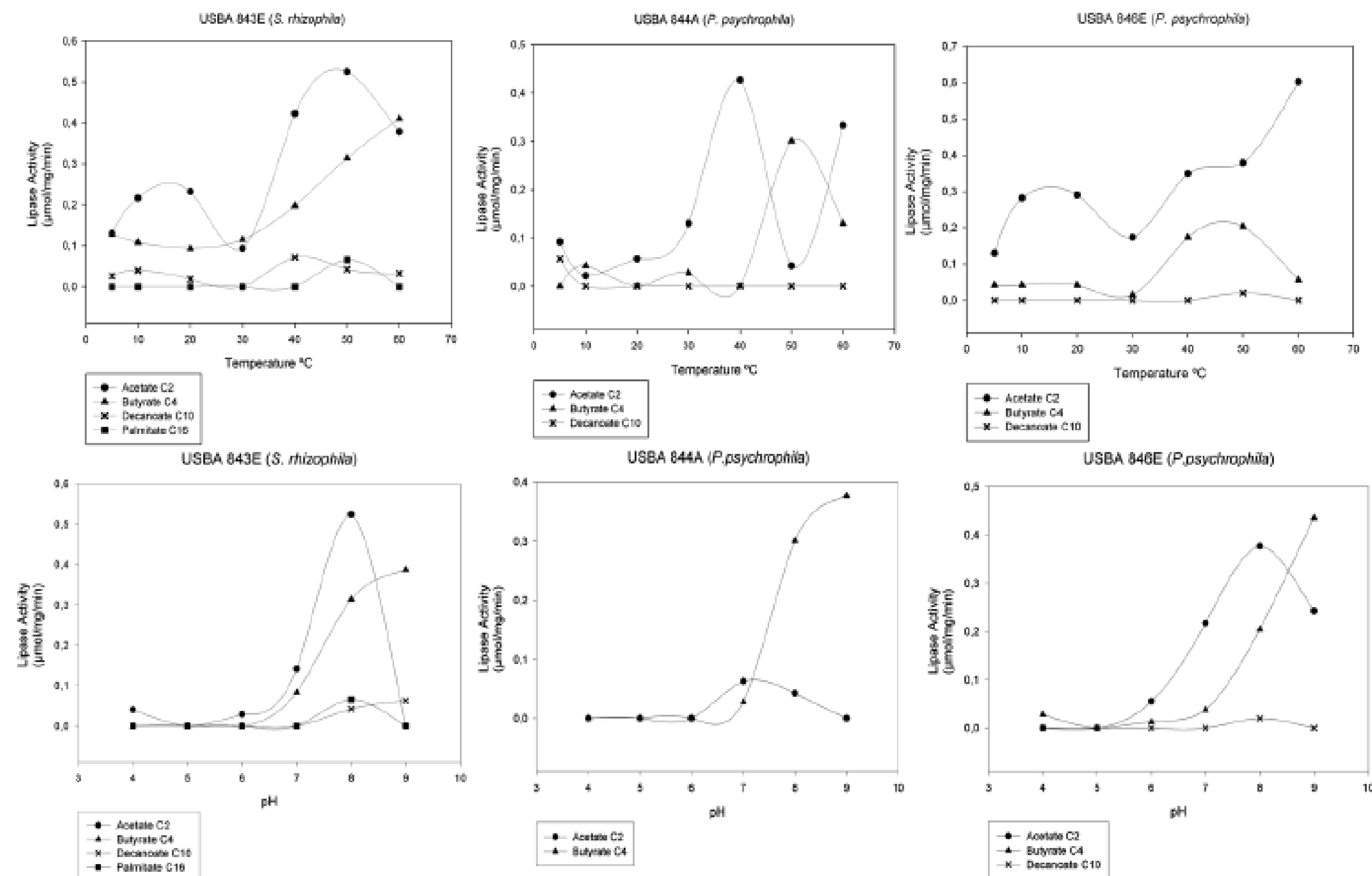

Figure 3 Lipolytic activities from the isolates USBA 843E, USBA 844A and USBA 846E at different temperatures and pH.

DISCUSSION

Enrichment of environmental samples with a substrate of interest aimed to expose microorganisms under a selection pressure. Here the "survival of the fittest" takes place under different physical, chemical and nutritional conditions (**Li *et al.*, 2009**). The adaptation of natural and complex microbial communities to specific conditions (e.g. degradation of olive oil) can increase the prevalence of microbes carrying special metabolic capacities such as lipolytic activity (**DeAngelis *et al.*, 2010**).

Pseudomonas and *Stenotrophomonas* were the most abundant genera recovered from the enrichments, and they also exhibited the largest hydrolysis halos. This shows how their ability to metabolize the given substrate allowed them to increase their population compared to other lipolytic bacteria. **Bardgett *et al.* (2005)** reported that the diversity tends to fall when the better-adapted microorganism dominates the community. In our case, the better-adapted microorganisms with the ability and required enzymes to degrade the non-conventional substrate, dominated the enrichment culture communities.

Furthermore, when we compared the lipolytic isolates from both soils, we found greater species diversity in the glacier enrichment culture than the one obtained from paramo. This is counter intuitive to expectation if we consider that the optimal environmental conditions, in terms of temperature, humidity and nutrient availability were present in the paramo soil (**Buytaert *et al.*, 2006**). In this case it is necessary to take into account that the enrichment method involved changes in the dynamics of the community. This could be caused predominantly by the microbial interactions within the bioreactor and the selection pressure in olive oil (**Bardgett *et al.*, 2005**).

The phyla Proteobacteria and Actinobacteria (with *Dermacoccus nishinomiyaensis* as the only isolate) identified in the glacier enrichment cultures have been reported from other cold environments (**Cheng & Foght, 2007; Steven *et al.*, 2007; Neufeld *et al.*, 2004; Shivaji *et al.*, 2006**). However, some studies also indicate the presence of different taxa such as Bacteroidetes and Firmicutes. They also report the presence of psychrophilic and lipolytic bacteria including *Pseudomonas, Aeromonas, Burkholderia* and *Ralstonia*, (**Yuan *et al.*, 2010; Joseph *et al.*, 2008; Hemachander & Puvanakrishnan, 2000**) which were also found in our enrichment cultures.

One particular finding in our study was the isolation of bacteria with different colony morphotypes. This polymorphism complicated the macroscopic description of some of the isolates, but after finishing the bacterial identification, this change was observed only for the *Pseudomonas* isolates. **Workentine *et al.* (2010)** described that *Pseudomonas* can change its colony morphotype depending on the culture conditions.

In terms of lipolytic activity, this potential for *Pseudomonas fragi* and *Ralstonia picketti* was confirmed by the large hydrolysis halos produced in the fatty substrates (Fig 1 and 3). These species have been previously reported for their lipolytic activity and stability at low temperatures (**Joseph *et al.*, 2008; Hemachander & Puvanakrishnan, 2000**). The other two isolates with interesting activity were *Pseudomonas psychrophila* and *Stenotrophomonas rhizophila*. They were chosen to perform enzymatic activity assays, not only for the production of hydrolysis halos, but also for their "novelty" value. **Bai & Rai Vittal. (2014)** studied the relationship between protease and lipase production with quorum sensing molecules in *P. psychrophila* PSPF19. Their objective was to inhibit the exoenzyme production. Therefore, they never characterized the lipases or proteases produced by the strain PSPF19. The closest report of the lipolytic activity in *P. psychrophila*, was performed by **Yumoto *et al.* (2001)** with *Pseudomonas fragi* lipase, which has a close phylogenetic association with *P. psychrophila*. In conclusion, our study is the first to date to perform the preliminary characterization of its lipolytic activity.

In 2002 **Wolf et al.** did the whole characterization of *Stenotrophomonas rhizophila*, a novel plant-associated bacterium. In their study they reported that the three strains evaluated showed a high antifungal activity and no lipase or β-glucosidase production, which differs from the characteristics observed from the 13 strains of *S. maltophila*. In addition, the growth rate of *S. rhizophila* at 4°C was 6.25 times higher than the observed with *S. maltophila*, which lead us to predict an even higher potential for the purified enzyme of *S. rhizophila* at low temperatures. After this publication, **Hasan-Beikdashti *et al.* (2008)** optimized the culture conditions to produce the *S. maltophila* lipase to hydrolyze capsaicin and produce vanillylamine, which has antibacterial and anti-inflammatory properties. **Li *et al.*** in 2013 and 2016 cloned and characterized the lipases: CGMCC 4254 (novel cold-active and organic solvent-tolerant lipase), and the GS11 (cold-active, solvent-tolerant and alkaline) also from *S. maltophila*. Nevertheless, this is the first study that reports the lipolytic activity in *S. rhizophila*.

The optimal conditions for the three evaluated strains were 50°C and pH 8 (Fig 8). Those temperatures and pH conditions have been already evaluated in *Pseudomonas aeruginosa* (**Dharmsthiti *et al.*, 1998**), *Pseudomonas fluorescens* (**Kulkarni *et al.*, 2002**), *Pseudomonas sp. INK1* isolated in the Antarctic (**Park *et al.*, 2012**). Our possible "novel" alkaline enzymes should be purified and tested

for a complete characterization in order to explore all the potential in terms of stability, substrate specificity and possible applications.

CONCLUSION

Exposing microorganisms to specific conditions in order to screen for capabilities that are not usually explored in non-conventional environments is a useful tool to discover biotechnological applications. Our study reports the first preliminary characterization of the lipolytic activity of *Stenotrophomonas rhizophila* and *Pseudomonas psychrophila*. Further studies to purify and completely characterize their lipolytic enzymes, will help us to understand all the potential offered by these "novel" lipases/esterases, that could be used for future applications. Our results demonstrate the vast opportunities offered by extreme environments for exploration and bioprospecting of different metabolic activities of interest.

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ANTIMICROBIAL ACTIVITY OF MARINE MICROALGAE ISOLATED FROM MOROCCAN COASTLINES

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ABSTRACT

The present investigation aimed to study antimicrobial activities in marine microalgae, screened from Moroccan coastlines. Ethanolic extracts were prepared from the microalgae and evaluated each against the bacteria: *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, the yeast *Candida albicans* and the fungus *Aspergillus niger*. The highest antibacterial activity was found in the extract of *Tetraselmis* sp. which exhibited an inhibitory effect against the three bacteria with a MIC of 2.6 to 3.0 mg extract per mL culture. Extracts from the other microalgae: *Dunaliella Salina*, *Nannochloropsis gaditana*, *Dunaliella* sp., *Phaeodactylum tricornutum* and *Isochrisis* sp. each showed inhibitory activity against *E. coli* or *P. aeruginosa* with a MIC of 2.6 to 4.3 mg extract per mL. The extract of *N. gaditana* also inhibited *S. aureus* growth. However, the extracts from the microalgae, *Chaetoceros* sp. and *Chlorella* sp. showed no effect under the applied experimental conditions. All the tested extracts inhibited the growth of *C. albicans*; the highest activity was obtained from *N. gaditana* with a MIC of 4.0 mg extract per mL culture. *Aspergillus niger* appeared to be resistant to the effect of the extracts. The observed antimicrobial activities were linked to the contents of the extracts in fatty acids, carotenoids and phenolic compounds. In conclusion, the studied microalgae could be considered as a potential natural source of bioactive compounds with antimicrobial activities.

Keywords: Marine microalgae, antimicrobial activity, phenolic content, carotenoids, fatty acids

INTRODUCTION

There has been increasing demand for new antimicrobial compounds in response to continuous evolution of microbial pathogens in antibiotic-resistance. Marine environment has been considered among the most promising sources of antimicrobial compounds, as numerous sea organisms produce bioactive metabolites in response to environmental stress and develop chemical strategy for defense and survival (**Mhadhebi *et al.*, 2012**). A large number of new active antimicrobial compounds have been isolated from marine sources. But, the majority of these compounds has not been yet characterized (**Sanmukh *et al.*, 2014**).
Marine microalgae constitute attractive sources of novel and active metabolites, comprising proteins, enzymes, pigments and polyunsaturated fatty acids (PUFA) that could be exploited in pharmaceutical, food, feed and cosmetic industries (**Mendes *et al.*, 2003; Cardozo *et al.*, 2007; Surendhiran *et al.*, 2014**). Compounds with pharmaceutical characteristics, as antioxidative, antiinflammatory, antimicrobial or antitumoral properties, have been identified; some of them have been in the clinical trial state (**Guedes *et al.*, 2011; Kwak *et al.*, 2014**). Antimicrobial activities are among the most researched features in natural extracts. They have been attributed to different compounds, including, indoles, terpene derivatives, acetogenins, phenols, fatty acids and hydrocarbons (**Bhakuni and Rawat, 2005; Santoyo *et al.*, 2009**).
Selected examples from the studied antimicrobial activities in microalgae are summarized in Table 1. However, to our knowledge, there are no data available on the antimicrobial potential of microalgae, isolated from the Moroccan coastlines. In order to evaluate this potential, nine microalgae, collected from these coastlines and identified, were extracted with ethanol; the collected extracts were examined against microbial targets. The obtained results are described hereafter, and discussed with consideration to the previously determined contents of the microalgal extracts in fatty acids, carotenoids and phenolic compounds (**Maadane *et al.*, 2015**).

Table 1 Extracts and compounds from microalgae with antimicrobial activity

Microalgal species	Active extracts/compounds	Target microorganisms	References
Phaeodactylum tricornutum	Eicosapentaenoic acid	*Listonella anguillarum* *Lactococcus garvieae* *Staphylococcus aureus* *Vibrio* sp.	(**Desbois *et al.*, 2009**) (**Smith *et al.*, 2010**)
Dunaliella salina	Faty acids	*Staphylococcus aureus* *Candida albicans*	(**Herrero *et al.*, 2006**)
Haematococcus pluvialis	Short chain fatty acids (butanoic acid, methyl lactate)	*Escherichia coli* *Staphylococcus aureus Candida albicans*	(**Santoyo *et al.*, 2009**)
Chloroccum humicola *Spiriluna platensis*	Pigments	*E. coli* *Staphylococcus aureus* *Bacillus subtilis* *Streptococcus* sp. *Bacilus* sp. *Pseudomonas* sp.	(**Bhagavathy *et al.*, 2011**) (**Muthulakshmi *et al.*, 2012**)

		Staphylococcus sp. *E. coli*	
Anabena sphaerica *Oscillatori alimentica* *Spirulina platensis*	Polyphenols	*E. coli* *Staphylococcus aureus*	**(Klejdus *et al.*, 2010)** **(Hetta *et al.*, 2014)**
Pithophora oedogonium	Ethanolic extract	*Salmonella* *Staphylococcus* sp.	**(Danyal *et al.*, 2013)**
Tetraselmis suecica	Fatty acids	*Vibrio cholerae*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* sp. *Proteus* sp. *Streptococcus pyogens*, *Staphylococcus aureus*, *Bacillus megaterium* *Bacillus subtilis*	**(Dooslin Mercy Bai and Krishnakumar, 2013)**

MATERIALS AND METHODS

Microalgae and culture conditions

Nine marine microalgae were selected for this study from the collection of Moroccan Foundation for Advanced Science, Innovation & Research (MAScIR, Rabat). They were identified (**Maadane *et al.*, 2015**) as: *Nannochloropsis gaditana*, *Dunaliella salina*, *Dunaliella* sp., *Phaedactylum tricornutum*, *Isochrysis* sp., *Navicula* sp., *Chaeotoceros* sp., *Chlorella* sp. and *Tetraselmis* sp. Culturing conditions of the microalgae were previously described (**Maadane *et al.*, 2015**). Briefly, batch cultures were realized in 5 L flasks containing sterile natural seawater, enriched with F/2 medium nutrients; the cultures were agitated by air bubbling at 25 °C, under continuous illumination at intensity of 150 µmol.m^{-2}.sec^{-1}; microalgal biomasses were harvested by centrifugation, freeze-dried and stored at -20° until use.

Extraction of microalgal substances

Crude extracts from the microalgae were prepared by extracting 1 g of dried biomass with 100 ml of ethanol for 3 h at room temperature, in the darkness. The extraction was repeated twice for each alga; collected extracts were combined into one sample. The samples were then filtered and concentrated under reduced pressure in a rotary evaporator. The resulting concentrated extracts were stored at -20°C until use.

Extract antimicrobial activity evaluation

The microalgal extracts were tested against five microorganisms, including two Gram-negative bacteria: *Escherichia coli* (ATCC-8739), *Pseudomonas aeruginosa* (ATCC-9027), one Gram-positive bacterium, *Staphylococcus aureus* (ATCC-6538), the yeast *Candida albicans* (ATCC-10231) and the mold *Aspergillus niger* (ATCC-16404). Spores of the mold were harvested into sterile distilled water from monoconidial cultures, developed on potato dextrose at 24°C during 7 days.
The minimum inhibitory concentrations (MICs) of the extracts were determined against all the tested microorganisms using the broth microdilution method (**Scorzoni *et al.*, 2007**). Microbial samples were prepared by dilution with growth media to obtain inocula at 10^5 colony forming units (CFU) per mL culture. The test cultures were performed in Muller Hinton broth for bacteria, or in Sabouraud dextrose broth for *C. albicans* or *A. Niger*. The culturing media were supplemented with 0.5% Tween-20. Samples of the extracts were prepared by different dilutions in DMSO, and tested at a final concentration ranging from 0.5 to 5 mg extract per mL culture. Microbial growth was allowed in 96-well micro-titration plates by dispensing into each well 180 µL of microbial culture and 20 µL of microalgal extract sample at various concentrations. The final DMSO amount, being less than 4%, was without growth inhibition effect. Sample blanks were prepared for all the extracts by adding 20 µL of each extract sample to 180 µL of Mueller Hinton (or Sabouraud dextrose) broth medium. The plates were incubated at 37°C, 24 h for the bacteria and 48 h for *C. albicans* or *A. niger*. The antibiotics, chloramphenicol and amphotericin B were used as positive controls against the bacteria or *C. albicans* and *A. niger*, respectively. The viability of the examined microorganisms was assessed by measuring absorbance of cultures at 600 nm against the Mueller Hinton or the Sabouraud dextrose broth, using a Multiscan Spectrophotometer (Thermo-Fisher Scientific Inc). Assays were carried out in triplicate, and repeated twice. Microbial growth inhibition was expressed in percentage term according to the equation:

$$\left[1 - \left(\frac{A_{sample} - A_{sample\ blank}}{A_{control}}\right)\right] \times 100$$

$A_{control}$: absorbance of the cullture control; A_{sample}: absorbance of the culture test sample; $A_{sample\ blank}$: absorbance of the extract sample in the nutrient broth.

RESULTS AND DISCUSSION

The antimicrobial activity level in the ethanolic extracts of the examined microalgae varied depending on the targeted microorganism and the microalgal strain (Table 2). The extract of *Tetraselmis* sp. exhibited an antimicrobial activity against both Gram-positive and Gram-negative bacteria with the lowest MIC values, ranging from 2.6 to 3.0 mg per mL culture. Meanwhile, the ethanolic extract from *Dunaliella salina* showed an antibacterial activity against only the Gram-negative bacteria, *E. coli* and *P.* aeruginosa. However, the extracts from *N. gaditana*, *Dunaliella* sp. *P. tricornutum*, and *Isochrysis* sp. showed relatively moderate antibacterial activities at the used extract concentrations. *Chlorella* sp. and *Chaetoceros* sp. did not exhibit any inhibitory activity against the tested bacteria, even at the concentration of 5.0 mg extract per mL.

Table 2 Antimicrobial activities of ethanolic extracts of microalgae, isolated from Moroccan coastlines.
MIC values are presented as means ± SD (n = 6). The antimicrobial activity was determined in a culture assay of 0.2 mL, composed of 0.18 mL medium and 0.02 mL extract sample.

Microalgae	MIC (mg extract per mL culture)				
	E.coli	*P. aeruginosa*	*S. aureus*	*C. albicans*	*A. niger*
Tetraselmis sp.	2.6±0.2	2.9±0.9	3±0.1	>5	-
Dunaliella Salina	3.4±0.4	2.6±0.8	-	>5	-
Nannochloropsis gaditana	3.4±0.2	>5	4.5±0.6	4.0±0.6	-
Chlorella sp.	-	-	-	>5	-
Dunaliella sp.	>5	4.3±0.1	>5	>5	-
Navicula sp.	>5	>5	>5	>5	-
Phaeodactylum tricornutum	4.3±0.1	>5	>5	4.7±0.1	-
Chaetoceros sp.	-	-	-	>5	-
Isochrisis sp.	3.5±0.2	>5	-	>5	-

>5: MIC is expected to be higher than 5 mg extract per mL culture. -: absence of antimicrobial effect at the highest examined extract concentration, 5 mg per mL culture.

Only, *N. gaditana* and *P. tricornutum* showed an activity against *C. albicans* (Table 2). Extracts of the other microalgae had weak activity against this yeast, as at the maximal concentration used in the test, being 5.0 mg per mL culture, the growth inhibition was less than 50%. Therefore, the MICs of these extracts against *C. albicans* were expected to be higher than this concentration. Paradoxically, no antifungal activity was observed in all the extracts against *A. niger* at the tested concentrations.

As noticed under the applied culture conditions, the MICs of the positive controls are in mean values: 0.085, 0.145 and 0.571 mg chloramphenicol per mL culture for respectively *S. aureus*, *E. coli* and *P. aeruginosa*. The MIC of amphotericin against *C. albicans* was much higher, being 1.6 mg per mL culture. For the microalgae that exhibited antimicrobial activities, the registered MICs, which were of 2.6 to 4.7 mg extract per mL (Table 2) are by comparison to those of the antibiotics higher. However, they might be pharmaceutically significant, if the crude state of the tested extracts was considered, as these extracts contained different substances, and only some of them had inhibitory effect. The registered data were in fact concordant with literature-published observations, concerned with the antimicrobial activities of microalgal extracts, as those of *Chaetoceros mulleri* (**Mendiola *et al.*, 2007**), *Chlorella vulgaris* (**Plaza *et al.*, 2012**) and *Nostoc* sp. (**Salem *et al.*, 2014**).

The antimicrobial activities described above could be attributed to different compounds, comprising those previously determined (**Maadane *et al.*, 2015**): fatty acids, carotenoids and phenolic compounds. Fatty acids, which constitute major parts of the extracted biomasses, are particularly considered because their antimicrobial effects have been long recognized (e.g., **Galbraith *et al.*, 1971**; **Desbois *et al.*, 2009**; **Cakmak *et al.*, 2014**).

The extract from *Tetraselmis* sp. contains high amounts of oleic acid (*cis*-9-octadecenoic acid) (48.8 % of the ethanol-extracted fatty acids), linoleic acid (*cis*, *cis*-9,12-octadecadienoic acid) (36.4 %) and palmitic acid (hexadecanoic acid) (18.6%). These fatty acids were determined as major components in the ethanolic-extract or hexanic-extract of *Dunaliella salina* (**Herrero *et al.*, 2006**), and reported to be responsible, in a main part, of the antimicrobial activity, exercised against *E. coli*, *S. aureus*, and *C. albicans*. Besides, the antibacterial activities observed in the algal species, *Nostoc spongiforme, Oscillatoria tenius and Chlorococcus* sp. were linked to their contents in fatty acids (**Suresh *et al.*, 2014**). Considering these literature-data, the antibacterial activity of *Tetraselmis* sp. (Table 2) can be linked, for a major part, to its content in palmitic, oleic and linoleic acids.

The analysis of the ethanolic extract from *D. salina* showed that this microalga contains the highest amount of ethanol extractable PUFA, being 76.9 % of which linolenic acid (*all-cis*-9,12,15-octadecatrienoic acid) constitutes a high part (45.3%) (**Maadane *et al.*, 2015**). According to Lee *et al.* (2002), the antimicrobial activity of linolenic acid was high against Gram-positive bacteria, but low against Gram-negative bacteria. The ethanolic extract of *D. salina* (Table 2) showed antibacterial activity against the Gram-negative bacteria, *E. coli* and *P. aeruginosa* and no activity against the Gram-positive bacterium *S. aureus*. If this activity was mainly attributed to the linolenic acid which was found to be abundant in the tested extract, then our observations regarding *S. aureus* are in contrast to those by Lee *et al.* (2002).

The extract from *N. gaditana* was shown to contain important amounts of palmitoleic acid (*cis*-9-hexadecenoic acid) (28%), palmitic acid (24.1%), oleic acid (15.3%) and eicosapentaenoic acid (14%). Also, *P. tricornutum* extract was shown to be rich in these acids. Their antimicrobial activities against bacteria and *C. albicans* (Table 2) might be due for a great part to these dominant fatty acids, in agreement with published observations (**Surendhiran *et al.*, 2014**), concerned with the antibacterial activity of the C16-C20 fatty acids-rich extract from *N. oculata*. Especially, eicosapentaenoic acid, dominant in extracts of *P. tricornutum* must have considerable antimicrobial effect (**Desbois *et al.*, 2009**).

Astaxanthin, a carotenoid pigment, was demonstrated to have a significant effect against both Gram-negative and Gram-positive bacteria (**Ushakumari and Ramanujam, 2013**). Besides, different extracted carotenoids from microalgae, comprising *D. salina* (**Herrero *et al.*, 2006**) and *Chlorococcum humicola* (**Bhagavathy *et al.*, 2011**) have been reported to possess important antimicrobial activities, effecting Gram-positive and Gram-negative bacteria and *C. albicans*. According to our previous data (**Maadane *et al.*, 2015**), only the ethanolic extracts from *Dunaliella* sp., *P. tricornutum* and *Tetraselmis* sp. contained carotenoids at significant amounts, being 10.8, 6.3, and 4.6 mg per g extract, respectively. Consequently, the observed antimicrobial activities of these extracts could be due not only to their fatty acids, but also to their carotenoids, in concordance with the referenced data. Besides, the ethanol extracts from *N. gaditana*, *Tetraselmis* sp. and *P. tricornutum* contained high content of phenolic compounds (polyphenols), being 32, 25.5 and16.8 mg, expressed in gallic acid equivalent per g extracted biomass, respectively. These extracts exhibited the highest antimicrobial activity (Table 2) which could be attributed, for a part, to their phenolic compounds in agreement with published observations (e.g., **Pane *et al.*, 2015**).

Bioactive compounds released by microalgal cells are either bactericidal or bacteriostatic (**Falaise *et al.*, 2016**). Their action mechanisms are still poorly understood. However, action modes have been suggested for growth inhibition or killing of bacteria by some of the functional molecules (**Shannon and Abu-Ghannam, 2016**). In this, phytochemicals, comprising microalgal substances, may act by inducing cellular membrane perturbations, interference with certain microbial metabolic processes, modulation of signal transduction or gene expression. Free fatty acids could initiate peroxidative processes, and preclude the synthesis of bacterial fatty acids (**Zheng *et al.*, 2005**; **Desbois and Smith, 2010**). Besides, free fatty acids might interact with cellular membranes of microbial cells, causing leakage of molecules from these cells, reduction of their nutrient uptake or inhibition of their respiration (**Suresh *et al.*, 2014**).

In the present study the antimicrobial activities of the studied microalgal extracts were attributed to their contents of fatty acids, carotenoids and polyphenols, as discussed above. These substances probably act together, either in an independent or synergistic manner. Whatever their action mode, the data, described here, demonstrated the presence of pharmaceutically promising antibacterial compounds in the screened microalgae from the Moroccan costlines.

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MOLECULAR IDENTIFICATION AND ANTIBIOGRAM OF *Enterococcus* spp. ISOLATED ON ENTEROCOCCUS SELECTIVE DIFFERENTIAL (ESD) MEDIA FROM MEAT, MEAT PRODUCTS AND SEAFOOD IN LIBYA

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ABSTRACT

This study was conducted to investigate the presence of *Enterococcus* spp. in meat, meat products and seafood. A hundred and four samples were randomly collected from different geographic localities in Libya. The samples were subjected to microbiological analysis for enumeration and isolation of *Enterococcus spp.* by conventional cultural and molecular identification using PCR and partial sequencing of 16S rDNA techniques. Out of 104 samples, 73 (70.2%) isolates were found to be enterococci based on their cultural characteristics on ESD medium. However, out of 36 samples subjected to molecular identification, only six isolates were confirmed to be *Enterococcus* spp. using PCR and partial sequencing of 16S rDNA technique. All enterococci strains tested for their antibiotic sensitivity profiles showed high percentage of multi-resistance phenotype. These results can be used for further studies on enterococci as an emerging food borne pathogen and its role in human infection in Libya and would suggest that meat, meat products and seafood might play a role in the spreading of enterococci through the food chain with antimicrobial resistance characteristics.

Keywords: 16S rDNA, antibiogram, enterococci, food, Libya

INTRODUCTION

Enterococcus spp. is a genus of lactic acid bacteria of the phylum Firmicutes that possess Lancefield group D antigen as some of streptococci. Enterococci are Gram-positive cocci, often occur in pairs (diplococci) or short chains bacteria of the gastrointestinal tract of healthy human intestinal flora (**Aarestrup *et al.*, 2001**). Enterococci are able to survive in extremes of temperature (5 to 60 °C), pH (4.6 to 9.9) and high sodium chloride (6.5% w/v) (**Murray, 1990**). They are capable of growth in the presence of bile salts (40% w/v) (**Fisher and Phillips, 2009**) and they commonly occur in foods, especially those of animal origin such as meat and milk (**Giraffa, 2003**).
Previously, all streptococci of fecal origin that produce group D antigen were considered as enterococci (**Hartman *et al.*, 2001**). Molecular biology studies (including oligonucleotide cataloging of 16S rRNA, DNA-DNA and DNA-rRNA hybridization), combined with physiological studies showed more detailed classification (**Schleifer and Kilpper-Bälz, 1987**). Members of this genus are: *E. avium*, *E. casseliflavus*, *E. durans*, *E. faecalis*, *E. faecium*, *E. gallinarum*, *E. hirae*, *E. malodoratus* and *E. mundtii* (**Hartman *et al.*, 2001**).
Previous studies have shown that meat and meat products represent a continuous supply of commensal bacteria, including enterococci (**Choi and Woo, 2013; Sharifi *et al.*, 2013; Sparo *et al.*, 2013**). *E. faecalis* and *E. faecium* are common commensal organisms in the intestines of humans were shown to be the predominant isolates in raw meat (beef and pork carcasses) (**Knudtson and Hartman, 1993**). Meanwhile, *E. faecalis* was the most frequent isolate among the Gram-positive cocci found in chicken meat (**Turtura and Lorenzelli, 1994**). In processed meat, the presence of enterococci reflects the extent of initial fecal contamination (**Holley *et al.*, 1988**).
Enterococci are recognized as opportunistic human pathogens and lately have distinguished themselves as major nosocomial pathogens causing bacteremia, endocarditis, urinary tract, central nervous system, intra-abdominal and pelvic infections (**Franz *et al.*, 1999**). In addition, enterococci can be also used as an enteric contamination indicator (**Foulquie Moreno *et al.*, 2006**).
Enterococci are also known for their capability to exchange genetic information by conjugation (**Dunny, 2007**) and may spread antibiotic resistance genes among non-pathogenic organisms (**Cocconcelli *et al.*, 2003; Fisher and Phillips, 2009**). Thus, there is a concern about their presence in uncooked fermented meats because of the contribution they may have to the baseline level of antibiotic resistance in other genera and the potential for transfer of antibiotic resistant bacteria from the indigenous animal microflora to the human gastrointestinal tract (**Mathur and Singh, 2005**), also leading causes of highly antibiotic-resistant and hospital-acquired infection (**Aarestrup *et al.*, 2001**). Enterococci are recognized as opportunistic human pathogens, and as indicator for fecal contamination. Due to lack of good hygienic practice in the Libyan slaughterhouses and meat retail markets, therefore, the objectives of this study were to evaluate the presence of enterococci in meat, meat products of different animal species and seafood from different Libyan localities and for their antibiotic resistance profiles.

MATERIAL AND METHODS

Collection and preparation of samples

A total of 104 samples (Table 1) included: raw meat samples (51), meat products (30) and seafood (23), were randomly collected from different cities in Libya (Tripoli, Regdalin, Janzour and Tobruk). The samples were packed in sterile plastic bags, stored in an insulated icebox and transferred as quickly as possible to Food Hygiene and Control Laboratory Department, Faculty of Veterinary Medicine, University of Tripoli. All samples were subjected to *Enterococcus* spp. microbiological enumeration and isolation techniques. Decimal dilutions, culturing and enumeration techniques were performed according to the methods described by the American Public Health Association (APHA) (**Downes *et al.*, 2001**). Briefly, 25 g from each sample was aseptically transferred into a sterile stomacher bag (Seward Medicals, UK) and homogenized (Stomacher 400, Seaward Medicals, UK) with 225 mL of sterile peptone water 0.1% (w/v) (Park Scientific, UK) at 230 rpm for 2 min.

Enumeration and isolation of *Enterococcus* spp.

Enumeration and isolation of enterococci were performed using enterococci selective differential agar medium (ESD) (**Efthymiou *et al.*, 1974**). ESD plates were seeded by surface spreading of 0.1 mL of appropriate tissue homogenate serial dilutions and then incubated at 37 °C for 24 h. ESD plates were examined for the presence of either magenta, round, 2-3 mm diameter colonies (*E. faecalis*), or white, round, 2-3 mm diameter colonies (*E. faecium*), or pink, round, 2-3 mm diameter colonies (*E. intermediate*). Isolates were identified to the species level by using API 20 Strep system (bioMérieux®, France).

Identification of enterococci by PCR and partial sequencing of 16S rDNA
DNA extraction and amplification of 16S rDNA

DNA extraction of enterococci isolates was performed by GF-1 bacterial DNA extraction kit (Cat. # GF-BA-100, Vivantis, Malaysia) as described in a previous study (**Azwai *et al.*, 2016**). The 16S rDNA was amplified using the universal oligonucleotides primers forward: S-D-Bact-0341-b-S-17 5'-CCTACGGGNGGCWGCAG-3' and Reverse: S-D-Bact-0785-a-A-21 5'-GACTACHVGGGTATCTAATCC-3' (**Herlemann *et al.*, 2011**).

Electrophoresis, gel extraction and DNA sequencing

The amplified 16S rDNA PCR fragment (464 bp) was excised from the gel and the DNA was purified using GF-1 Ambi Clean kit (Cat. # GF-GC-100, Vivantis, Malaysia) as described in previously (**Azwai *et al.*, 2016**). The purified 16S rDNA amplicons underwent cycle sequencing with Big Dye® Terminator v1.1 kit (AB Applied Bioscience, TECHNE, TC-512, USA) and were sequenced on four capillary ABI PRISM® 3130-Avant Genetic Analyzer at IZSLER Istituto Zooprofilattico Sperimentale Della Lombardia e dell'Emilia Romagna, Brescia, Italy. Sequences were assembled and edited using the SeqMan module within Lasergene package, (DNA Star Inc., Madison, WI, USA). The obtained consensus sequences were subjected to BLAST search both at NCBI (http://www.ncbi.nlm.nih.gov/pubmed) and at 16S bacterial cultures Blast Server for the identification of prokaryotes (http://bioinfo.unice.fr/blast/).

Antibiogram of isolated strains

Inoculum Preparation

Upon confirmation by PCR and partial sequencing of 16S rDNA gene isolated strains of enterococci were preserved by freezing at -80 °C in vials containing Brain Heart Infusion broth (BHI, Difco, Michigan, USA) supplemented with 30% (v/v) glycerol. To propagate the culture, frozen vial was thawed at room temperature, and 0.5 mL of thawed culture was transferred to 5 mL of BHI broth and incubated for 24 h at 37 °C. The inoculum was prepared from the second transfer of that culture (0.5 mL) to another 5 mL of BHI broth and incubated for 16 – 18 h at 37 °C. After the overnight incubation Muller Hinton agar plates (Oxoid, Hampshire, UK) were surface swabbed, then the selected antibiotic discs were dispensed and lightly pressed onto the inoculated agar surface according to (**Coyle, 2005**) then incubated at 37 °C for 24 h.

Antibiotic assay

The selection of antibiotics was based on their common use in food animal practice and included: (oxytetracyclin (30 µg), streptomycin (10 µg) and vancomycin (30 µg)). The antibiotic discs were purchased from Oxoid with the exception of the enrofloxacin (5 µg), amoxicillin (25 µg) obtained from Arcomex Arab (Medical Diagnostics CO., Amman, Jordan), while colistin (10 µg), doxycycline (30 µg), gentamycin (10 µg), erythromycin (10 µg), were obtained from Mast Diagnostics (Mast group ltd., Merseside, UK). The clear zones around antibiotic discs that has no growth, referred to as the zone of inhibition, were measured and scored as sensitive, intermediate (reduced susceptibility) or resistant according to the Clinical and Laboratory Standards Institute guidelines (**CLSI, 2015**).

RESULTS AND DISCUSSION

Isolation and enumeration of *Enterococcus* spp.

One hundred and four samples from various regions of Libya comprising raw meat (51), meat products of different species (30) and seafood (23) were tested for the presence of *Enterococcus* spp. by using ESD medium (Table 1). *Enterococcus* spp. were isolated from the samples of raw meat: beef 12/17 (70.5%), camel 13/22 (59%) and chicken meat 11/12 (91.6%) respectively, and from the samples of seafood: fish 5/13 (38.4%) and shrimp 3/6 (50%) respectively, with counts ranged from 8.7x10 to $4.2x10^4$ CFU/g and the most common isolate was *E. faecalis*. No isolate was detected from clam samples. As for meat products, isolation rate of *Enterococcus* spp. on ESD agar plates from 30 samples of meat products of different animal species was 100%, except for beef burger, that was 87.5% (7/8) with counts ranged from $7x10^3$ to $6.8x10^6$ CFU/g. The maximum mean count of enterococci was recorded in chicken burger $3.8x10^6$ CFU/g; while the minimum mean count was in shrimp $1.1x10^3$ CFU/g (Table 1). The occurrence of *Enterococcus* spp. was 87.5% in beef burger with counts ranging from $1.7x10^5$ to $1.4x10^6$ CFU/g and the mean counts was $7.6x10^5$CFU/g, meanwhile, in beef kebab the isolation rate was 100% with counts ranging from $2x10^4$ to $1.8x10^5$ CFU/g and the mean count was $9x10^4$ CFU/g (Table 1). Detection of enterococci in chicken burger was 100% with counts ranging from $7.7x10^5$ to $6.8x10^6$ CFU/g with a mean counts $3.8x10^6$ CFU/g. While, in ground chicken the rate was 100% with counts ranging from $9x10^3$ to $8x10^4$ CFU/g and the mean counts was $4.5x10^4$ CFU/g.

Table 1 Comparison between growth on ESD medium and partial sequencing of 16S rDNA technique for identification of *Enterococcus* spp.

Type of Sample	No. of Samples	No. of Suspected *Enterococcus* spp. Growth on ESD (%)	Average Count (*CFU/g*) of *Enterococcus* spp. on ESD	No. of Sequenced Isolates	No. of Positive *Enterococcus* spp. by 16S rDNA Sequencing
Raw meat					
Beef	17	12 (70.5)	2.2×10^4	4	None
Camel meat	22	13 (59)	1.6×10^4	4	None
Chicken meat	12	11 (91.6)	4×10^3	4	None
Clam	4	0	-	-	-
Fish	13	5 (38.4)	4.4×10^3	2	None
Shrimp	6	3 (50)	1.1×10^3	2	None
Meat products					
Chicken burger	8	8 (100)	3.8×10^6	4	3
Chicken kebab	2	2 (100)	9×10^4	2	None
Chicken sausage	2	2 (100)	9×10^4	2	None
Beef burger	8	7 (87.5)	7.6×10^5	4	1
Beef kebab	2	2 (100)	9×10^4	2	1
Beef sausage	2	2 (100)	8×10^4	2	None
Ground beef	2	2 (100)	9×10^3	2	None
Ground chicken	4	4 (100)	4.5×10^4	2	1
Total	**104**	**73 (70.2)**		**36**	**6**

Identification of enterococci spp. by PCR and sequencing of partial 16S rDNA gene

A total of 36 (16 raw meat samples and 20 meat products samples) randomly selected isolates (36 out of 73 isolates were found to be enterococci based on their cultural characteristics on ESD medium) were sent for partial sequencing of 16S rDNA (464 bp) of enterococci strains using the universal oligonucleotides primers (FOR.: S-D-Bact-0341-b-S-17 and REV.: S-D-Bact-0785-a-A-21) (Fig. 1). Only six isolates (16.6%) (Table 2) were identified as *Enterococcus* spp. These isolates of enterococci were all isolated from meat products (beef burger, beef kebab, ground chicken and chicken burger) (Table 3).

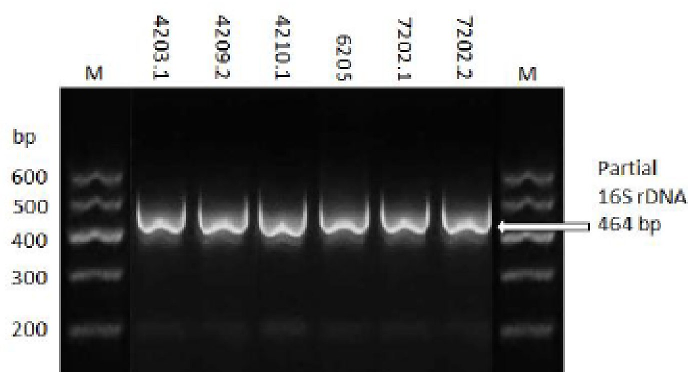

Figure 1 Representative gel of partial amplification of 16S rDNA (464 bp) products of isolated Enterococci strains using the universal oligonucleotides primers. First and last lanes contain DNA marker (M).

Table 2 Convential and molecular identification of suspected *Enterococcus* spp. in different meat products samples (CFU/g)

Type of Sample	Suspected Growth of *Enterococcus spp.* on ESD	No. of Suspected Isolates Growth on ESD	No. of Sequenced Isolates	No. of Positive *Enterococcus* spp. by 16S rDNA Sequencing
Chicken burger	*E. intermediate*	4	2	0
	E. faecalis	4	2	3
Chicken kebab	*E. intermediate*	1	1	0
	E. faecalis	1	1	0
Chicken sausage	*E. intermediate*	1	1	0
	E. faecalis	1	1	0
Beef burger	*E. intermediate*	3	2	0
	E. faecalis	4	2	1
Beef kebab	*E. intermediate*	1	1	1
	E. faecalis	1	1	0
Beef sausage	*E. intermediate*	1	1	0
	E. faecalis	1	1	0
Ground beef	*E. intermediate*	1	1	0
	E. faecalis	1	1	0
Ground chicken	*E. intermediate*	2	1	0
	E. faecalis	2	1	1
Total		**29**	**20**	**6**

Table 3 Identity of suspected isolate after sequencing by blast NCBI

Blast NCBI	Identity (%)	Isolate Code	Suspected Isolate on ESD	Type of Sample	Storage Condition	Source
Enterococcus durans	100	4203.1	Enterococci	Beef burger	Frozen	Suqaljuma, Tripoli
Enterococcus faecium	100	4209.2	Enterococci	Chicken burger	Frozen	Salaheldin Tripoli
Enterococcus durans	100	4210.1	Enterococci	Beef kebab	Chilled	Salaheldin Tripoli
Enterococcus faecalis	100	6205	Enterococci	Ground chicken	Chilled	Suqaljuma, Tripoli
Enterococcus faecium	100	7202.1	Enterococci	Chicken burger	Frozen	Abusetta, Tripoli
Enterococcus durans	100	7202.2	Enterococci	Chicken burger	Frozen	Abusetta, Tripoli

Antibiotics Resistant Phenotype

The results (Table 4) showed testing of the six confirmed enterococci isolates from meat products against nine antimicrobial agents (amoxicillin, colistin, doxycycline, enrofloxacin, erythromycin, gentamycin, oxytetracyclin, streptomycin and vancomycin). Antibiotic resistance profile showed that, *E. durans* found in beef burger and *E. faecium* found in chicken burger were resistant to five out of nine antibiotics (55.5%). Meanwhile, *E. durans* from beef kebab and *E. faecalis* from chicken burger both were resistant to seven out of nine antibiotics (77.7%). On the other hand *E. faecalis* from ground chicken showed resistance to eight out of nine (88.8%), lastly *E. durans* from chicken burger was resistant to six out of nine (66.6%). In conclusion, enterococci isolates exhibited resistance to at least five out of nine (55.5%) of the tested antibiotics. All six enterococci isolates (100%) were resistant to colistin. While five out of six tested isolates (83.3%) were resistant to amoxicillin, enrofloxacin, erythromycin and streptomycin. Resistance to oxytetracyclin and doxycycline was recorded among 66.6% of the isolates. However, only two isolates (33.3%) were resistant to gentamycin and vancomycin (Table 4).

Table 4 Sensitivity of six strains of enterococci to nine antibiotics

Enterococci Strains	Isolate Codes	Antibiotic Discs (mm)									R%	S%
		E (10 µg)	CO (10 µg)	AMO (25 µg)	S (10 µg)	ENR (5 µg)	DOX (30 µg)	Gen (10 µg)	Van (30 µg)	OT (30 µg)		
Enterococcus durans	4203.1	20 (S)	(R)	(R)	(R)	9 (R)	25 (S)	12 (R)	20 (S)	21 (S)	**55.5**	**44.5**
Enterococcus faecium	4209.2	12 (R)	(R)	(R)	(R)	12 (R)	23 (S)	16 (S)	19 (S)	21 (S)	**55.5**	**44.5**
Enterococcus durans	4210.1	11 (R)	(R)	(R)	(R)	13 (R)	10 (R)	15 (S)	17 (S)	(R)	**77.7**	**22.3**
Enterococcus faecalis	6205	(R)	(R)	16 (S)	(R)	16 (R)	(R)	(R)	(R)	(R)	**88.8**	**11.2**
Enterococcus faecium	7202.1	(R)	(R)	(R)	(R)	13 (R)	10 (R)	15 (S)	17 (S)	(R)	**77.7**	**22.3**
Enterococcus durans	7202.2	(R)	(R)	(R)	20 (S)	20 (S)	(R)	23 (S)	(R)	(R)	**66.6**	**33.4**
R%		**83**	**100**	**83**	**83**	**83**	**66.6**	**33.3**	**33.3**	**66.6**		
S%		**17**	**0**	**17**	**17**	**17**	**33.4**	**66.6**	**66.6**	**33.4**		

(S): Sensitive, (R): Resistant, E: erythromycin, CO: colistin, AMO: amoxicillin, S: streptomycin, ENR: enrofloxacin, DOX: doxycycline, Gen: gentamycin, Van: vancomycin, and OT: oxytetracyclin.

DISCUSSION

Enterococcus spp. are widely distributed in nature and are associated with the spoilage of meat and meat products (**Hugas *et al.*, 2003**). Current study was conducted to isolate *Enterococcus* spp. from 104 samples of different meat, meat products of different animal species and seafood, collected from various geographical places in Libya. This study reported the presence of *Enterococcus* spp. in most meat and all local un-heat treated meat products samples except one sample of beef burger by conventional cultural method. Generally, the incidence of *Enterococcus* spp. all over the collected samples of raw meat was 70.5% (36/51) and seafood was 34% (8/23). However, the incidence rate of enterococci in meat products was 96.6% (29/30); this high incidence in meat products could be attributed to low hygienic practice and cross contamination during preparation of such products. The results showed that the contamination with *Enterococcus* spp. in meat of different animal species and meat products was higher than that in seafood. The higher values could be as a result of contamination from the processing area, equipment used, also the means of transportation which was used in bringing the produce to the market centers and the hygienic practice employed by meat sellers and butchers. The meat during its preparation remains in the ground for a long time which creates a good environment for microbial pathogens to proliferate on it. On the other hand, seafood were sold at the seafood market freshly with better hygienic conditions that reduce the possibility from being contaminated (**Franz *et al.*, 2003**).

The occurrence of *Enterococcus* spp. in meat of different animal species and seafood (73) was in beef, camel, chicken, fish and shrimp 70.5%, 59%, 91.6%, 38.4 and 50% respectively, with counts ranging from $1.5x10^4$ to $6.8x10^6$ CFU/g (Table 1). The average counts of *Enterococcus* spp. in camel meat was $1.6x10^4$ $\pm 1.2x10^4$ CFU/g. **Hugas *et al.* (2003)** reported that the numbers of viable count of enterococci in contaminated beef, poultry and pork are usually in the range of 10^2 – 10^4 CFU/g. Meanwhile, our study did not detect enterococci among four examined samples of clam (bivalve shellfish). In contrary to **Montiel *et al.* (2013)** who found enterococci in all samples of clam examined with their densities generally higher in clams than sediment and water. Our result could be due to *Enterococcus* spp. were removed from hard shell clams by depuration occurred at the fish market where the samples were collected (**Love *et al.*, 2010**).

On the other hand, 30 samples of meat products revealed an incidence of 100% *Enterococcus* spp., except in beef burger was 87.5% (Table 1). The mean counts of enterococci were $2.2x10^4$ CFU/g in beef, $3.8x10^6$ CFU/g in chicken burger, $9x10^4$ CFU/g in chicken kebab and beef Kebab, chicken sausage and ground beef, $8x10^4$ CFU/g in beef sausage, and $4.5x10^4$ CFU/g in ground chicken. Our study revealed that, the highest enterococci count was in chicken burger $3.8x10^6$ CFU/g, however, the lowest count $1.1x10^3$ CFU/g was recorded in shrimp. The most common enterococci recorded in our investigation in meat products were *E. durans*, *E. faecalis* and *E. faecium*, while, (**Jahan *et al.*, 2013; Sadeghifard *et al.*, 2015**) reported *E. faecalis* as a predominant isolate in all meat samples. In agreement with our findings, Naas *et al.* (2009a,b) recorded high enumeration of enterococci in all tested samples that included beef burger and beef sausage at rate of $2x10^7$ and $9x10^6$ CFU/g respectively. As for molecular confirmation only six out of 36 randomly selected enterococci isolates were identified and confirmed by partial sequencing of 16S rDNA. (8.2%) were confirmed as *Enterococcus* spp. in particular *E. durans*, *E. faecalis* and *E. faecium* (Table 3).

Enterococci raise major concern during the last decades, as they are becoming one of the most important nosocomial infections causing serious illnesses in human. The presence of *Enterococcus* spp. in foods may act as reservoir of antibiotic resistance genes (**Valenzuela *et al.*, 2009**). The susceptibility of enterococci isolates to different antibiotics was tested (Table 4) and the highest incidence of resistance was recorded to colistin (100%), colistin is a last-resort antibiotic in both animals and humans, this antibiotic is used against particularly dangerous types of multi resistant bacteria that can withstand many other antibiotics. The existence of such isolates in the food chain of humans is of a great concern not only to public health but also because of the ease of resistance gene transfer to other bacteria. Lower resistance rates (83%) were recorded against erythromycin, amoxicillin, streptomycin and enrofloxacin while it was (66.6%) to oxytetracyclin and doxycyclin. Only 33.3% of the isolates were resistant to vancomycin and gentamicin, similar results were recorded by **Jahan *et al.* (2013)**. Vancomycin resistant enterococci (VRE) are nosocomial pathogens that have been detected in environmental habitats including soil, water and wildlife faces. The spread of opportunistic pathogens harboring VR genes beyond hospitals into community is a potential threat to public health as vancomycin is used as last-resort against many infections. Most of the isolated enterococci strains were resistant to more than five antibiotics out of nine (55.5%). In the contrary to **Fracalanzza *et al.* (2007)** who found overall percentages of antimicrobial resistant of isolates were: 31.2% to tetracycline, 23.8% to erythromycin, 11.3% to streptomycin, 4.3% to chloramphenicol, 3.9% to gentamicin, 1.4% to enrofloxacin and 0.4% to ampicillin. In another work, **Klibi *et al.* (2013)** studied enterococci strains isolated from meat samples that showed 14% resistance to streptomycin and 100% to streptomycin and tetracycline.

CONCLUSION

In conclusion, our findings demonstrated the presence of *Enterococcus* spp. in meat, meat products of different animal species and seafood. Vancomycin resistant enterococci were also isolated from local meat products sold in different cities in Libya. Moreover, conventional cultural methods on ESD medium were less significant than using the molecular techniques as partial sequencing of 16S rDNA techniques for identification of enterococci. Only six enterococci isolates cultured on ESD medium were confirmed to be *Enterococcus* spp. by PCR and partial sequencing of 16S rDNA. The occurrence of resistant strains of enterococci in food of animal origin should be considered as important threat to public health.

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IN SILICO STUDIES ON THE EFFECT OF GRISEOFULVIN ON TUBULIN PROTEIN OF *CRYPTOCOCCUS NEOFORMANS* AND ITS *IN VITRO* VALIDATION

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ABSTRACT

Griseofulvin is a well known drug against dermatophytes. It is particularly prescribed for an infection called scrap ringworm or tinea capitis. In general, griseofulvin inhibits the tubulin protein that is responsible for the cell division. Not much is known about the effect of griseofulvin on *Cryptococcus neoformans*. Therefore, the authors made an effort to check the activity of griseofulvin against it.

The webservers (T-Coffee, Bluues simulation and CASTp) and software (Autodock 4.0) have been used in order to determine the activity of griseofulvin against the beta subunit of tubulin protein of *C. neoformans*. The results obtained from the *in silico* studies show a high affinity of griseofulvin towards the beta chain of tubulin protein. The negative value of binding energy (-9.02 kcal/mol) also shows that the complex is thermodynamically favourable and stable. These *in silico* results were further validated by MIC assay showing 74.1% inhibition of *C. neoformans* (isolate no. 5) against griseofulvin.

The present study reports that apart from dermatophytes, the drug has significant effect on *C. neoformans* and it may be used in the combinatorial therapy against the same.

Keywords: *Cryptococcus neoformans*; Griseofulvin; *In silico; In vitro* validation

INTRODUCTION

Cryptococcus neoformans is commonly known for causing cryptococcosis and cryptococcal meningitis. Most commonly the infection of *C. neoformans* starts with lungs but patients having advanced immune suppression end up with meningitis (**Centers for disease control and Prevention, 2014**). The general treatment for cryptococcosis in patients suffering from asymptomatic or mild to moderate pulmonary infections is fluconazole. This is slightly different from the treatment plan of those who have severe lung infections in which amphotericin B is prescribed in combination with flucytosine (**Centers for disease control and Prevention, 2014**). But in the recent past, some isolates of *C. neoformans* have shown resistance against these drugs and patients have witnessed relapse or failure on treatment with flucytosine, fluconazole and amphotericin B (**Lopez-Jodra et al., 2000**).

Griseofulvin is an antifungal drug known to be produced by 3 species of *Penicillium* viz. *P. patulum, P. griseofulvum and P. janczewskii* (**Drugs.com, 2015**). It has been used for the treatment of human and animal dermatophytic infections. The reason for its success against dermatophytes is due to its peculiar pharmacological property that after oral administration it is localized in the keratinized cells of skin, hairs and nails. The growth of the dermatophytes parasitizing these cells is thus inhibited and removed by desquamation (**Drugs.com, 2015**). Many sensitivity studies have been done on the activity of griseofulvin against superficial fungal infections (**Millikan, 2016**). But anticryptococcal activity of griseofulvin has not been reported yet.

Many studies advocate the *in vitro* endorsement of the *in silico* results, owing to the technical limitations of bioinformatics approach. For instance, **Nyarady et al (2005)** validated the results of *in silico* prediction of epitopes by a multipin ELISA. Similarly, the mechanism of serine hydroxymethyltransferase (SHMT) inhibition by pemetrexed was validated by *in vitro* assays and the calculated interaction energy of pemetrexed in the active site of SHMT and the corresponding predicted binding energy were found to be in good agreement with the values of K_d and Ki obtained in Isothermal Titration Calorimetry (**Daidone et al., 2011**).

The general mode of action of griseofulvin is to interact with tubulin molecule in the fungal cells (**Ronnest et al., 2012**). Hence, in the current manuscript the activity of griseofulvin against the tubulin protein of *C. neoformans* has been tested *in silico* and validation of the same has been done through the minimum inhibitory concentration assay (MIC) following the Clinical and Laboratory Standards Institute (**CLSI, 2008**) guidelines.

MATERIALS AND METHODS

Retrieval of the structure of griseofulvin and tubulin protein

The structure of griseofulvin (Drug data bank ID: DB00400) was obtained from drug data bank (http://www.drugbank.ca/) whereas reference structure of tubulin of *Sus scrofa* (wild boar) was obtained from the Protein data bank (PDB) (PDB ID: 1TUB). The sequence of beta chain of tubulin of *C.neoformans* was obtained from National Center for Biotechnology Information (NCBI) (Accession number: XP_568244.1). Modelling was done by Phyre^2 webserver with default parameters.

Determination of the active site region of the tubulin protein and docking studies

CASTp server, an online webserver which determines the structural pockets and cavities with the help of Delaunay triangulation and the alpha complex for shape measurements was used in order to determine the active site regions on the tubulin protein. The docking studies were performed through Autodock 4.0 (http://autodock.scripps.edu/). Further, Bluues simulation software (http://protein.bio.unipd.it/bluues/) was used to analyse the structural stability of the model. It calculates the total energy of the structure on the basis of generalized Born atom radii.

In vitro studies of griseofulvin against isolates of *C. neoformans*

Drug griseofulvin under the brand name of GRISOVIN-FP was obtained from GlaxoSmithKline and amphotericin B (AMB) (brand name AMPHOTRET) was obtained from Bharat Serums and Vaccines Limited.

Micro-organisms

One reference strain (Ref 1431), two environmental isolates (NCBI accession no. KJ175192 and KJ175193) and five clinical isolates (4CI2, 49, CSF2, CNS and CNS45) of *C.neoformans* were used in the study.

Antimicrobial agent

A stock solution of griseofulvin in dimethyl sulfoxide (DMSO) at a concentration of 100% was prepared (**Brilhante et al., 2014**). Three controls viz. negative, vehicle (DMSO) and positive (amphotericin B), were used in the current experimental plan. The concentration range of griseofulvin against which the growth of the fungi has been tested is from 2 µg/ml to 1.024 mg/ml..

Preparation of inoculum

The inocula of *C. neoformans* were prepared from the fresh cultures maintained in Sabouraud Dextrose Agar (SDA) medium for 48 hours at 37°C. Each fungal culture was added to 0.9% sterile saline solution, ensuring gentle scrapping of the fungal colonies with the aid of the inoculation loop to form a fungal suspension. The resulting fungal suspension was adjusted to 0.5 McFarland scale of turbidity. This suspension was further diluted in the ratio of 1:10 with RPMI medium to obtain the final concentration of $1.0\text{-}5*10^6$ CFU/ml.

In vitro susceptibility testing

The susceptibility testing of *C. neoformans* against griseofulvin was performed according to the guidelines issued by CLSI for the broth macro-dilution method M27-A3 (**CLSI, 2008**) with a few modifications. An incubation time span of 72 hours and 35° C temperature was followed in the experiment. Additionally, all the samples were tested in triplicate.

Determination of MIC

MIC of all isolates of *C.neoforams* was determined using broth macro-dilution method (**CLSI, 2008**). The optical density of all the samples was measured at 420 nm using spectrophotometer (ThermoScientific UV1).

Statistical analysis

The OD readings obtained at different concentrations of griseofulvin for each isolate were statistically analysed by F test followed by Holm Sidak test.

RESULTS

Griseofulvin is known to interact with tubulin protein which plays an important role during the cell division. The structure of the alpha beta tubulin of *C. neoformans* was unavailable, therefore, a multiple sequence alignment was performed between the eukaryotic tubulin protein sequences having known structures with *C.neoformans* tubulin sequence. T-Coffee software was used to carry out the multiple sequence alignment process (**Notredame et al., 2000**). A very highy sequence similarity (99%) was found between the tubulin protein of *C.neoformans* and *Sus scrofa* (Fig 1).

Figure 1 MSA of beta tubulin of *S. scrofa* and *C. Neoformans*

Henceforth, 1 TUB, the respective tubulin protein of *S. scrofa* was taken as reference in the current study. Top 10 active sites were determined on1TUB. These active sites were docked with griseofulvin. It was observed that the best binding energy was obtained at pocket ID: 167 having an area of 381.6 and a volume of 590.9 (Fig 2). The docked structure of griseofulvin on this pocket has been shown in fig 3 and the docking parameters have been provided in table 1.

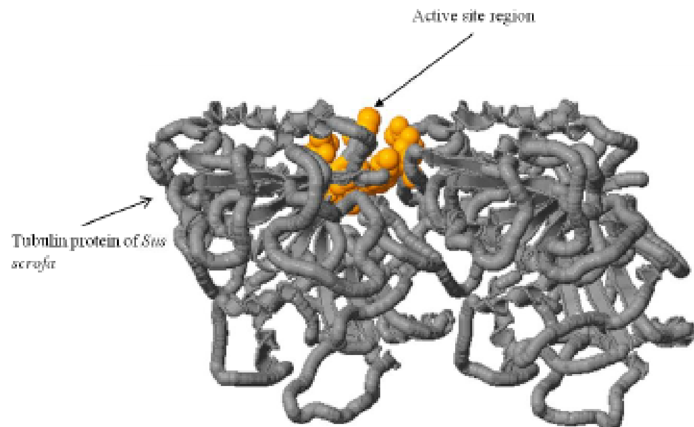

Figure 2 Prediction of active site of tubulin protein of *S. scrofa*

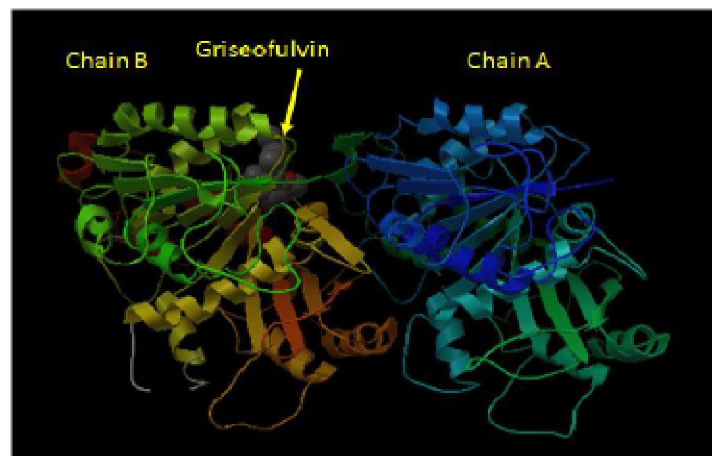

Figure 3 Best structure obtained after docking griseofulvin on tubulin protein of *S. scrofa*

Table 1 Parameters of the best docked structure of griseofulvin on tubulin protein (1TUB) of *S. scrofa*

Parameters	Values
Estimated Free Energy of Binding	-9.02 kcal/mol
Estimated Inhibition Constant (Ki)	243.84 nM (nanomolar)
Final Intermolecular Energy	-9.92 kcal/mol
vdW + Hbond + desolv Energy	-9.84 kcal/mol
Electrostatic Energy	-0.08 kcal/mol
Final Total Internal Energy	-0.45 kcal/mol
Torsional Free Energy	+0.89 kcal/mol
Unbound System's Energy	-0.45 kcal/mol

Estimated Free Energy of Binding or ΔG_{bind} is the most important parameter amongst various parameters shown in table 1. It is well known that only the negative ΔG_{bind} are energetically favourable. Here, the ΔG_{bind}= -9.02, hence this is energetically favourable and the resultant complex formed is thermodynamically stable. Docking result of the reference protein suggested that griseofulvin had a higher affinity to bind the beta chain of tubulin in comparison to its alpha chain therefore, suitable sequence of tubulin beta chain of *C.neoforamans* was searched for modelling it.. The modelled structure has been shown in fig 4.

Figure 4 Modelled structure of beta tubulin of *C. neoformans* obtained by Phyre[2] webserver

The obtained negative total energy assured that the model could be safely used for further docking analysis. The different parameters obtained through the Bluues software have been provided in table 2.

Table 2 Protein stability parameters predicted by Bluues software

Parameters	Values
Born self energy:	-16169.476521 (kJ)
Coulomb energy:	-109416.947909 (kJ)
Electrostatic solvation energy:	-4719.453945 (kJ/mol)
Total energy:	-112139.794202 (kJ/mol)

Again the best10 active sites for the modelled structure of beta tubulin were determined and griseofulvin was docked on all these 10 sites. It was observed that pocket ID 103 (active site region of beta tubulin of *C.neoformans*) having an area of 219.5 and a volume of 258.6 showed the highest binding energy when docked with griseofulvin (Fig 5 and Fig 6). The parameters related to the best docking structure obtained from Autodock 4.0 have been provided in table 3.

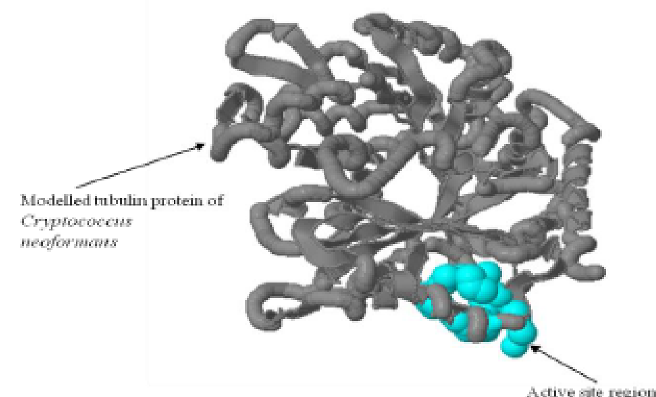

Figure 5 Prediction of active site on modelled tubulin protein of *C. neoformans*

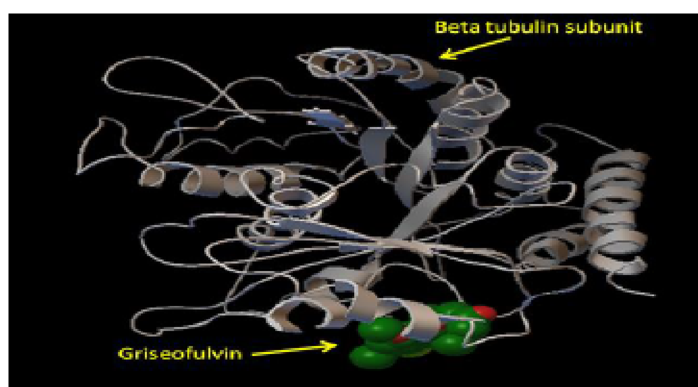

Figure 6 Best structure obtained of docking griseofulvin on beta tubulin subunit *C. neoformans*

Table 3 Parameters of the best docked structure

Parameters	Values
Estimated Free Energy of Binding	-7.05 Kcal/mol
Estimated Inhibition Constant (Ki)	6.75 uM (micromolar)
Final Intermolecular Energy	-7.95 kcal/mol
vdW + Hbond + desolv Energy	-7.94 kcal/mol
Electrostatic Energy	-0.01 kcal/mol
Final Total Internal Energy	-0.52 kcal/mol
Torsional Free Energy	+0.89 kcal/mol
Unbound System's Energy	-0.52 kcal/mol

Table 3 shows that ΔG_{bind}= -7.05 which is energetically favourable hence providing evidence that complex formed would be structurally favourable.

Further, MIC of griseofulvin against eight isolates of *C. neoformans* (one reference, two environmental and five clinical) was found to inhibit the growth of the strain 4CI2, CNS, Ref 1431, 9, 49, CSF2, 5 and CNS45 by 35%, 43%, 45%, 39.5%, 54%, 66.6%, 74.1% and 49% respectively on comparison with their respective controls (Table 4).

Table 4 MIC of griseofulvin against eight isolates of *C.neoformans*

Drug	Isolate	Range	MIC	% inhibition at this MIC
Griseofulvin	*C. neoformans* Ref 1431	64 µg/ml - 1.024 mg/ml	128 µg/ml	45%
	C. neoformans 4CI2	64 µg/ml - 1.024 mg/ml	1.024 µg/ml	35%
	C. neoformans CNS	64 µg/ml - 1.024 mg/ml	128 µg/ml	43%
	C. neoformans 9	64 µg/ml - 1.024 mg/ml	64 µg/ml	39.5 %
	C. neoformans 49	2-64 µg/ml	16 µg/ml	54%
	C. neoformans CSF2	64 µg/ml - 1.024 mg/ml	256 µg/ml	66.6%
	C. neoformans 5	64 µg/ml - 1.024 mg/ml	512 µg/ml	74.1%
	C. neoformans CNS45	64 µg/ml - 1.024 mg/ml	128 µg/ml	49%

DISCUSSION

Griseofulvin was the only drug available for treatment of *Tinea capitis*, a fungal infection caused by dermatophytes until the approval of terbinafine in 2007 (**Gupta and Summerbell, 2000; Seebacher et al., 2007**). It is known to inhibit microtubule assembly and the growth of the cells by inducing abnormal mitosis and blocking the cells at G_2/M phase of cell cycle (**Panda et al., 2005; Rebacz et al., 2007**). Tubulin is a major structural component of microtubule and consist of α and β subunits (**Luduena et al., 1977**). It has already been reported that griseofulvin does not disrupt the microtubules, (**Watson, 2004**) instead it interacts with tubulin or with one or more associated proteins of microtubules (**Sloboda et al., 1982; Roobol et al., 1977; Chaudhari and Luduena, 1996**).
In the present study, anticryptococcal activity of griseofulvin was performed against eight isolates of *C. neoformans* and significant (74.1%) inhibition was observed against isolate number 5. However, MIC of griseofulvin was found to be very high than MIC of amphotericin B but low toxicity and pharmacokinetic parameters like high elimination rate of griseofulvin than amphotericin B suggests that it could be a better drug.
In the present study, due to unavailability of structure of tubulin protein of *C.neoformans*, structure of tubulin alpha beta dimer of *S. scrofa* was taken as reference. Griseofulvin was found to efficiently bind with beta domain of tubulin. Ten griseofulvin binding sites on tubulin were also predicted. Unlike, **Rathinasamy et al. (2010)**, the binding site at Pocket ID:167 gave the best binding results. However, in case of modelled structure of beta tubulin of *C. neoformans* the best docking was obtained at Pocket ID:103.
Keeping in view, the ability of griseofulvin to inhibit mitosis in fungal cells, to stabilize microtubule dynamics and its high elimination rates, it can be suggested that the drug may be used in combination therapy for the treatment of cryptococcosis. Further, its low toxicity and weak binding to mammalian brain tubulin (**Wehland, 1977; Panda et al., 2005**) makes it safer for human use.
Henceforth, this study will certainly be helpful in designing more potent and specific analogues of griseofulvin against the tubulin domains of *C. neoformans* with least side effects to the host and will provide understanding about the uniqueness of binding site of griseofulvin.

CONCLUSION

Griseofulvin is a well-known drug against dermatophytes but its activity against *C. neoformans* is not known. The activity of griseofulvin has been tested against eight isolates of the same. *In silico* docking results have been validated by

minimum inhibitory concentration assay. The high inhibition in growth gives an understanding that griseofulvin is effective against *C. neoformans*. The results obtained in this study can be extended and griseofulvin can further be investigated for its effect in *in vivo* condition .Its use as combinatorial therapeutic is also suggested.

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CHARACTERIZATION AND ISOLATION OF PEPTIDE METABOLITES OF AN ANTIFUNGAL BACTERIAL ISOLATE IDENTIFIED AS BACILLUS AMYLOLIQUEFACIENS SUBSPECIES PLANTARUM STRAIN FZB42

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ABSTRACT

Some bacteria produce antimicrobial chemicals in their immediate environments. These antimicrobial agents are enzymes, polypeptides or non-protein organic compounds. In this study, a bacterial isolate that produces antifungal chemical(s) was isolated from an over incubated nutrient agar plate that was exposed to air. The bacterium is aerobic, Gram positive bacilli; capsule and endospore producing. It ferments glucose and sucrose but not lactose, galactose, mannitol and sorbitol; it is citrate, indole, methyl red and Voges Prauskauer negative. Using agar gel diffusion technique, the cell-free culture supernatant resulting from centrifugation of a six day bacterial culture showed antifungal activity against filamentous fungi but not yeasts. Heating the cell-free supernatant in 90 °C water bath and digestion with different proteases had no negative impact on the antifungal activity. A segment of 16S rRNA gene of the bacterial isolate was amplified. The nucleotide sequence of the amplicon was used to identify the bacterium as being very similar to *Bacillus amyloliquefaciens* subsp. plantarum strain FZB42. Five bacterial peptides were isolated and identified from cell free supernatant of the bacterial culture using a suite of techniques, including flash chromatography, HPLC, NMR and mass spectrometry. One of the five peptides has been previously reported in literature to possess antifungal activity.

Keywords: Antifungal; *Bacillus amyloliquefaciens*; peptide metabolites; preparative HPLC

INTRODUCTION

Different types of bacteria are sources of industrial enzymes and compounds that have antifungal or antibacterial properties. In a study of 1200 isolates of lactic acid bacteria by **Magnusson et al. (2003)**, 37 isolates showed inhibitory activity against different types of filamentous fungi and against the yeast *Rhodotorula mucilaginosa*. According to several studies, antifungal agents are produced by various species of *Bacillus*. In 2014, **Oyedele et al.** isolated *Bacillus subtilis, B. megaterium, B. licheniformis and B. pumilus* from different food sources. Nine strains of the isolated *B. subtilis* showed antifungal activities against a variety of filamentous fungi. Some of the other studies that showed antifungal activities of *Bacillus* species include **Islam et al., 2012** (*Bacillus subtilis*); **Munimbazi and Bullerman, 1998** (*Bacillus pumilus*); **Qazi et al., 2009** and **Pleban et al., 1997** (*B. cereus*); **Tendulkar et al., 2007, Trachuk et al., 1996** and **Takayanagi et al., 1991** (*B. licheniformis*); **Watanabe et al., 1990** (*B. circulans*). Other bacterial genera have also been reported to produce antifungal chemicals. As was reported by **Fguira et al., 2005** and **Rhee, Ki-Hyeong, 2003**, filamentous soil bacteria belonging to the genus *Streptomyces* exhibited antifungal and antibacterial activities resulting from production of non-protein compounds. **Lavermicocca et al. (2000)** isolated novel antifungal compounds from *Lactobacillus plantarum* associated with sourdough. Similarly, **Chernin et al. (1995)** showed that *Enterobacter agglomerans*, a soil-borne bacterium, was antagonistic to many plant fungi as a result of production of chitinolytic enzymes that hydrolyse fungal cell wall. The antifungal agents produced by different bacterial isolates were characterized (**Qazi et al., 2009**) or isolated, purified and characterized (**Fguira et al., 2005; Lavermicocca et al., 2000**). Most of the purified antifungal agents in the studies are polypeptides; some are chitinolytic enzymes while others are non-protein compounds, each with different properties. Such antifungal agents have potential uses in agriculture where they could serve as alternatives to chemical fungicides that are used to prevent plant fungal diseases of agricultural products and plants. Purified antifungal agents also have potential uses in cell culture and as food preservatives.

In the current study, nutrient agar plates were exposed to air and incubated at room temperature. One of these plates was overgrown with a filamentous fungus except around a bacterial colony where there was inhibition of fungal growth (**Figure 1**). The aims of this study are to characterize and identify the antifungal bacterial isolate and also the antifungal chemical produced by the bacterial isolate.

Figure 1 Nutrient agar plate showing a bacterial colony with antifungal activity indicated by a clear zone around the bacterial colony (arrow).

MATERIALS AND METHODS

Initial screening of efficacy of bacterial antifungal activity

The antifungal bacterium was subcultured on LB agar to obtain a pure culture. The efficacy of the bacterial isolate on fungal growth was done using agar diffusion method. Sabouraud dextrose agar plates were prepared and separate plates were inoculated with a wild yeast, *Candida albicans* and different types of filamentous fungi; the plates were incubated at 25 C. When fungal growth was evident on each of the plates, the bacterial isolate was then inoculated approximately one inch directly opposite the fungal growth; the plates were incubated further.

Characterization of bacterial antifungal chemical

The bacterial isolate was inoculated into 500 ml of LB broth and incubated at 25 °C in a shaker incubator. Cell free supernatant was obtained by centrifugation of the culture in a Beckman Model J2-21centrifuge using JA 14 rotor. The supernatant was further filtered using 0.45μM Nalgene disposable membrane filter ware. The resulting cell-free supernatant was stored at 4 C. Antifungal activity of the supernatant was determined using agar gel diffusion method.
The effect of temperature on the efficacy of the antifungal agent was carried out by incubating the cell-free supernatant at 40 °C, 50 °C, 70 °C and 90 °C for 30 minute. This was followed by efficacy test against filamentous fungus using agar gel diffusion.
Effect of enzyme treatment on the antifungal agent was done. The enzymes used were lipase, protease, proteinase K, pepsin and chymotrypsin. Culture supernatant was subjected to enzyme treatments for 1hr in a 37 °C water bath after which the enzyme was inactivated prior to determining the efficacy of the product on fungal growth. Efficacy was determined using agar gel diffusion method as stated earlier.

Morphological and biochemical characterization of the antifungal agent-producing bacterial isolate

Morphological characteristics of the bacterial isolate were determined using routine microbiological tests including Gram stain, endospore stain, capsule stain and motility test. Biochemical characterization of the bacterial isolate included the following tests: fermentation of different sugars, catalase production, oxidase test, MR-VP test, oxygen requirement test using fluid thioglycollate broth, indole production, citrate utilization, and production of exoenzymes for hydrolysis of starch, protein and lipid. All the microbiological tests were performed as are described by **Leboffe and Pierce (2008)**.

Identification of bacterial isolate

The bacterial isolate was identified by amplification and sequencing of segment of 16S rRNA gene using colony PCR and universal primers F-27 5'-AGAGTTTGATCMTGGCTCAG-3' and R1525 5'-AAGGAGGTGWTCCARCC-3'. The PCR conditions were denaturation at 94 °C for 5 min; 30 cycles: 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 1.5 min; final extension at 72 °C for 10 min. The PCR product was cloned into TA cloning vector following the manufacturer's protocol; this was followed by transformation. Recombinant plasmids were prepared from selected transformants using Qiagen Mini Plasmid Preparation kit and were quantitated using NanoDrop Spectrophotometer, ND-1000. DNA sequencing was done by Integrated DNA Technologies, Inc. The nucleotide sequence obtained was used to identify the bacterial isolate using the National Center for Biotechnology Information (NCBI) genomic BLAST.

Purification of antifungal metabolites

An extraction of the cell-free supernatant was performed by adding $CHCl_3$-CH_3OH (4:1). The mixture was stirred for 30 min and then transferred to a separating funnel. The bottom layer was drawn off and evaporated to dryness. The resulting organic component was further extracted with equal volume of CH_3OH-CH_3CN and hexanes; the two layers were drawn off, evaporated to dryness under vacuum and the dried extracts were weighed. This was followed by flash chromatography of the CH_3OH-CH_3CN extract. The extract was dissolved in $CHCl_3$ and transferred onto celite and allowed to dry. The celite was transferred and packed in a chromatography cartridge. At a 18 mL/min flow rate, a solvent gradient of 100% hexane to 100% $CHCl_3$ to 100% CH_3OH was flowed through the chromatography cartridge over 28 min. The sample was pooled into three fractions and evaporated to dryness. Fraction 2 was dissolved in 1:1 CH_3OH–dioxane and subjected to preparative HPLC. The mobile phase was a gradient of CH_3CN–H_2O with 0.1% formic acid. The gradient flowed at 15-20% CH_3CN over 15 min to 20% isocratic CH_3CN over 20 min at a 15 mL/min flow rate. The column used was an Atlantis Prep, OBD 19x250 mm column, No. 186004026. This yielded five isolated compounds.

Identification of antifungal metabolites

High resolution mass spectrometry (HRMS) was performed using a QExactive Plus (Thermo Fisher Scientific, San Jose, CA, USA). Resolution was 70,000, and MS/MS was performed with higher energy collisional dissociation (HCD) at 52.50. Additionally, the ^1H NMR (400 MHz; JEOL Ltd., Tokyo, Japan) was performed on the isolated compounds to confirm the structures.

RESULTS

Screening and characterization of antifungal metabolite

Streak of the bacterial isolate opposite *Candida albicans* and filamentous fungi showed that the antifungal metabolite secreted into the culture medium inhibited only filamentous fungi. **Figure 2** shows inhibition of a filamentous fungus by the bacterial isolate after incubation of the plate for four days. Cell-free supernatant obtained from centrifugation of the antifungal bacterial isolate in LB broth also showed growth inhibition of filamentous fungi (**Figure 3**). The results of heat treatment of the cell-free, culture supernatant showed that the antifungal metabolite is heat stable because the efficacy of the culture supernatant was not abrogated by the treatments (**Figure 4**). Treatment of the cell-free supernatant with the enzymes used in the studies produced no negative effect on the efficacy against filamentous fungi.

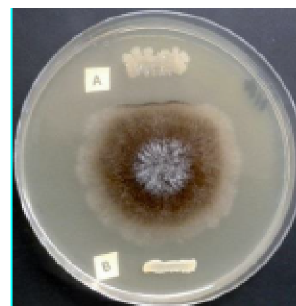

Figure 2. Inhibition of fungal growth after four day incubation at room temperature. Inhibition is shown as a straight line, by the antifungal bacterial isolate "A" inoculated on one side of fungal culture. Bacterium "B" is a non antifungal agent producing bacterium.

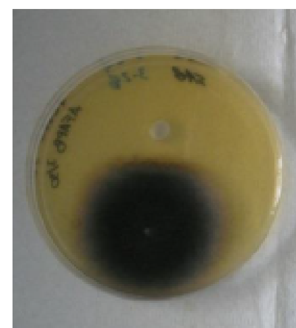

Figure 3. Sabouraud dextrose agar plate after two day incubation showing slight inhibition of filamentous fungal growth adjacent to a well that was filled with cell-free supernatant from the antifungal bacterial isolate culture. The inhibition is shown as a straight line and a decrease in the periphery of the fungal growth.

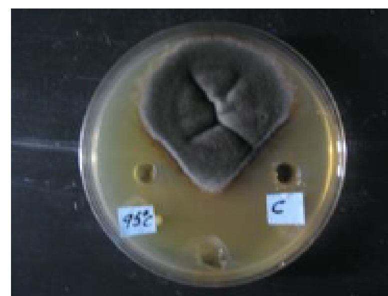

Figure 4. Inhibition of fungal growth opposite wells filled with cell-free culture supernatants (heated at 95 °C for 30 min and untreated control "C"). The plate was incubated at room temperature for more than 8 days during which the supernatants in the two wells were replenished several times. At the bottom of the plate is not a well but an accidental agar chip.

Morphological and biochemical characterization of antifungal bacterial isolate

The results of morphological and biochemical tests of the bacterial isolate are shown in Table I. The biochemical tests were carried out after approximately 24 - 48 hour incubation.

Table 1 Morphological and biochemical characteristics of the antifungal bacterial isolate.

Tests	Results
Gram Stain	Gram positive, bacilli
Endospores stain	Oval and centrally located
Capsule stain	Positive
Motility	Positive
Oxygen utilization	Aerobic
Catalase	Positive
Oxidase	Positive
Citrate utilization	Negative
Indole production	Negative
Methyl red	Negative
Voges Proskauer	Negative
Hydrolysis of:	
Starch	Positive
Protein	Positive
Lipid	Negative
DNA	Positive
Fermentation of:	
Glucose	Positive
Sucrose	Positive
Lactose	Negative
Galactose	Negative
Mannitol	Negative
Sorbitol	Negative

Identification of bacterial isolate.

A segment of 16S rRNA was amplified using universal primers F21 and R1525. The nucleotide sequence (1541 nucleotides) of the amplified segment of 16S rRNA gene of the bacterial isolate is shown in **Figure 5**. The DNA sequence was compared with NCBI gene bank database using BLAST algorithm. The result showed a 99% homology of the search sequence with *Bacillus amyloliquefaciens* subsp. plantarum strain FZB42. The nucleotide sequence was deposited in GenBank under accession number SUB1340497 AF-1 KU738862.

AAGGAGGTGATCCAGCCGCACCTTCCGATACGGCTACCTTGTTACGACTTCACCCCAATCATCTGTCCCACCTTCGG
CGGCTGGCTCCTAAAAGGTTACCTCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACA
AGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTGC
AGACTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGCTTAACCTCGCGGTTTCGCTGCCCTTTGTTCTGTCCAT
TGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACC
GGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCC
AACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGCCCCCGAAGGGGACGTCCTATCTCTA
GGATTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGT
GCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTG
CAGCACTAAGGGGCGGAAACCCCCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCT
GTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTTACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACA
TCTCTACGCATTTCACCGCTACACGTGGAATTCCACTCTCCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCT
CCCCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAGAAACCGCCTGCGAGCCCTTTACGCCCAATAATTCCGGAC
AACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGG
TGCCGCCCTATTTGAACGGCACTTRTTCTTCCCTAACAACAGAGCTTTACGATCCGAAAACCTTCATCACTCACGCG
GCGTTGCTCCGTCAGACTTTCGYCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTC
AGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTCGCCTTGGTGAGCCGTTACCTCACCRACTAG
CTAATGCGCCGCGGGTCCATCTGTAAGTGGTAGCCGAAGCCACCTTTTATGTCTGAACCATGCGGTTCARACAACC
ATCCGGTATTAGCCCCGGTTTCCCGGAGTTATCCCAGTCTTACAGGCAGGTTACCCACGTGTTACTCACCCGTCCGC
CGCTAACATCAGGGAGCAAGCTCCCATCTGTCCGCTCGACTTGCATGTATTAGGCACGCCGCCAGCGTTCGTCCTG
AGCCATGATCAAACTCT

Figure 5 Consensus nucleotide sequence of the amplified segment of 16S rRNA gene of the antifungal bacterial isolate.

Purification and identification of antifungal metabolites

The $CHCl_3$-CH_3OH extract from the cell-free supernatant yielded 280 mg. Flash chromatography on this extract produced three fractions that weighed 0.20 mg, 19 mg and 220 mg. While fraction three had the largest yield, analytical HPLC revealed that it primarily contained sugars not shown). Therefore only fraction 2 was pursued further, which led to the isolation of compounds 1-5. These compounds were tentatively identified using high resolution and tandem mass spectrometry (**Table 2**). The structures of the compounds were then confirmed using ^{1}H NMR (**Figures S1** {**Supplement 1**} through **S5** {**Supplement 5**}). All of the isolated compounds were small cyclic peptides (**Figure 6**); the characterization data for each compound were in good agreement with the literature (**Chen et al., 2009; Nakamura et al., 2006; Stark and Hofmann, 2005**).

Table 2 High resolution mass spectrometry (HRMS) and MS/MS data of the isolated compounds.

Compound	$[M + H]^+$		MS/MS Fragments
	Measured	Calculated	
1	195.1128	195.1128	195, 98, 70
2	197.1284	197.1285	197, 169, 154, 141, 124, 100, 98, 72, 70
3	211.1440	211.1441	211, 183, 154, 138, 114, 98, 86, 70
4	211.1440	211.1441	211, 183, 154, 138, 114, 98, 86, 70
5	245.1284	245.1285	245, 217, 172, 154, 120, 98, 70

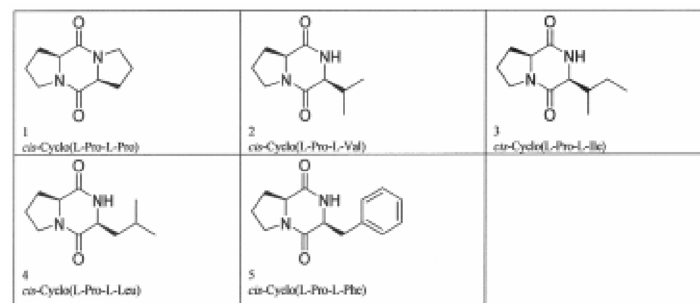

Figure 6 Structures of the five isolated metabolites of Bacillus amyloliquefaciens subspecies plantarum strain FZB42.

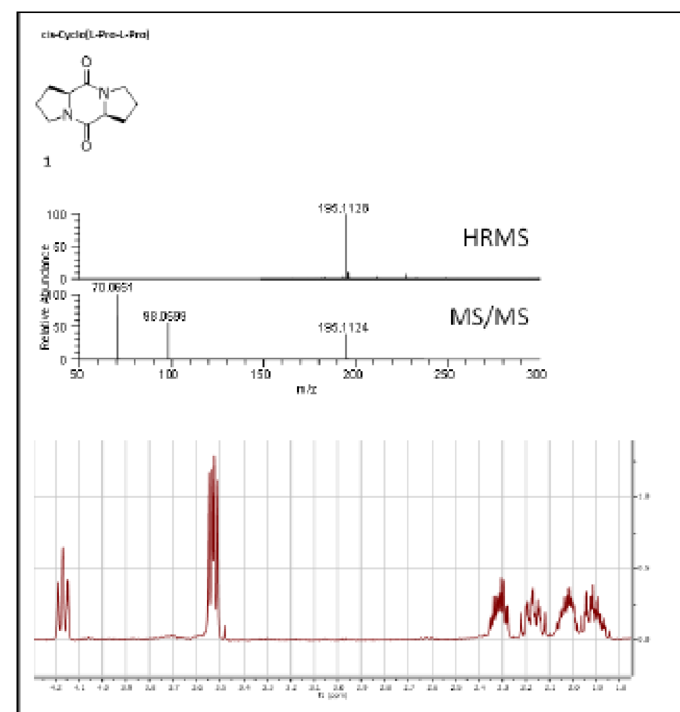

Figure S1 The structure of cis-Cyclo(L-Pro-L-Pro) (1) with the MS/MS fragmentation (HCD = 52.5) and 1H NMR (CDCl3, 400 MHz).

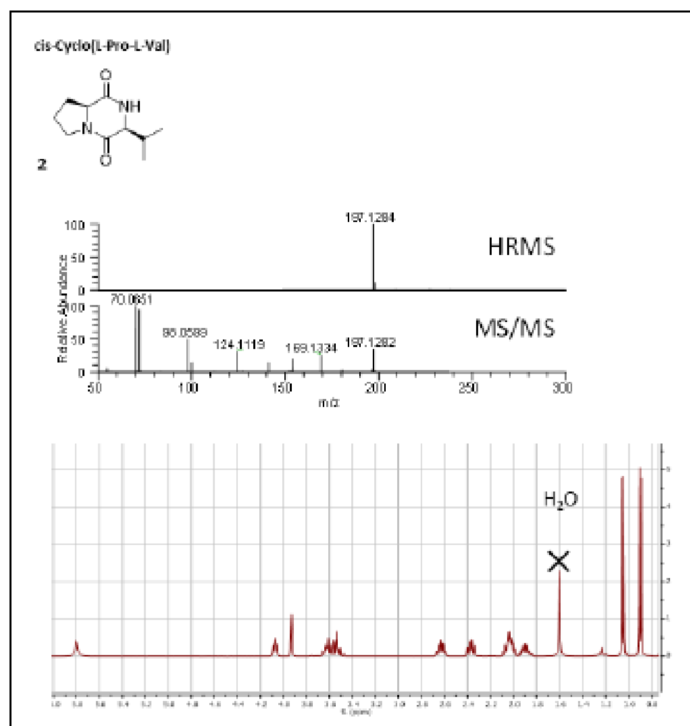

Figure S2 The structure of cis-Cyclo(L-Pro-L-Val) (2) with the MS/MS fragmentation (HCD = 52.5) and 1H NMR (CDCl3, 400 MHz).

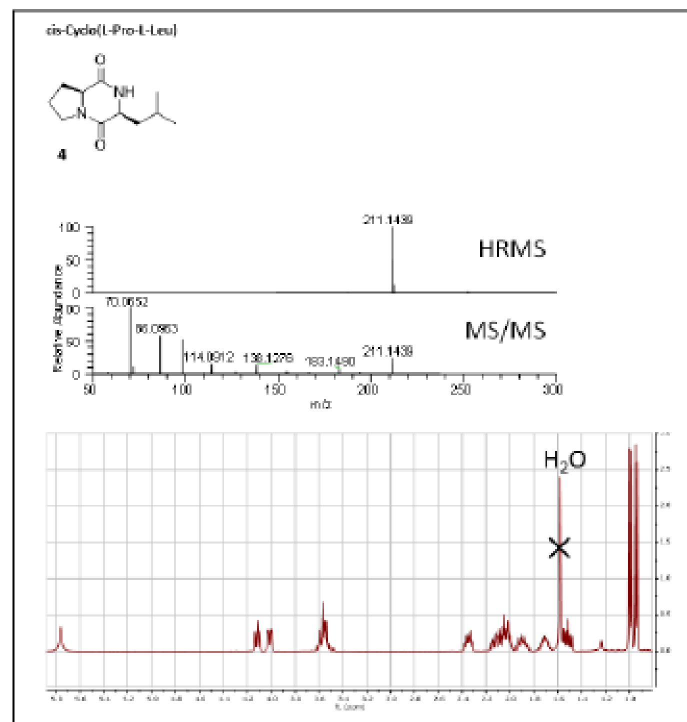

Figure S4 The structure of cis-Cyclo(L-Pro-L-Leu) (4) with the MS/MS fragmentation (HCD = 52.5) and 1H NMR (CDCl3, 400 MHz).

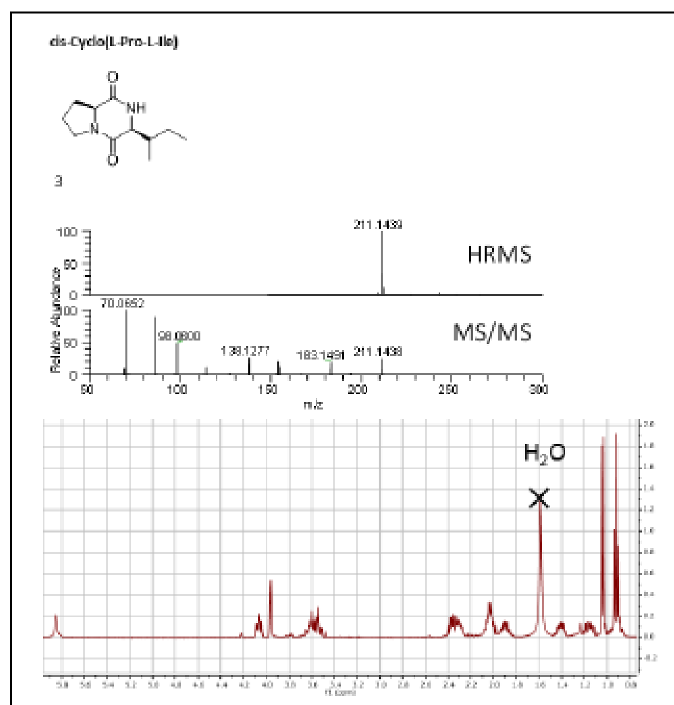

Figure S3 The structure of cis-Cyclo(L-Pro-L-Ile) (3) with the MS/MS fragmentation (HCD = 52.5) and 1H NMR (CDCl3, 400 MHz).

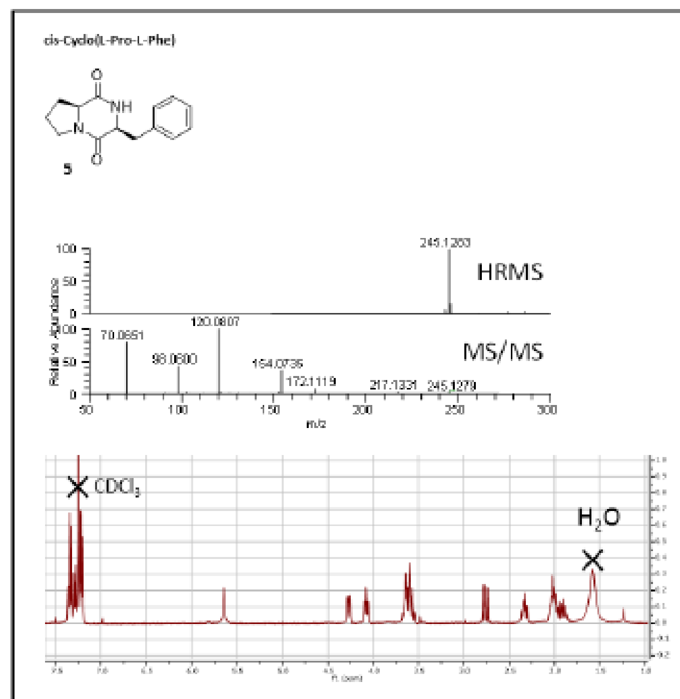

Figure S5 The structure of cis-Cyclo(L-Pro-L-Phe) (5) with the MS/MS fragmentation (HCD = 52.5) and 1H NMR (CDCl3, 400 MHz).

DISCUSSION

Microorganisms in different environments are in constant competition with each other for nutrients and other materials. As a result, some bacteria produce bacteriocins that inhibit or kill other bacteria species while some produce fungicidal or fungistatic metabolites. Similarly, some fungi produce antibacterial chemicals. According to **Meena and Kanwar (2015)**, "Bacillus genus is considered as the factories for the production of biologically active molecules that are potential inhibitors of growth of phytopathogens". This statement by **Meena and Kanwar** is supported by the current study and by the studies conducted by **Zhao et al. (2013)**, **Beric et al. (2012)**, **Chen (2012)**, **Islam et al. (2012)** and **Nair et al. (2002)**. Bacteria produce antifungal metabolites that include different types of peptides (**Meena and Kanwar, 2015**; **DeLucca and**

Walsh, 2000). In their study, **Munimbazi and Bullerman (1998)** reported that an isolate of *Bacillus pumilus* from a sample of dried fish was the source of an antifungal agent. Two different studies showed that *Bacillus amyloliquefaciens* GAI **(Arguelles-Arias et al., 2009)** and *Bacillus amyloliquefaciens* LJ02 **(Li et al., 2015)** produced secondary metabolites for the biocontrol of plant pathogen and for cucurbits powdery mildew, respectively. The antifungal activity of *B. amyloliquefaciens* GAI was due to cyclic lipopeptides surfactin, iturin A and fengycin. The antifungal agent produced by the bacterial isolate, *Bacillus amyloliquefaciens* subsp. plantarum strain FZB42, in the current study is similar to that produced by *B. pumilus* **(Munimbazi and Bullerman, 1998)** in their inhibitory effect on filamentous fungi and not yeasts, resistance to heat treatment and resistance to denaturation by hydrolytic enzymes. However, unlike the current study where the bacterial antifungal agent was isolated and identified, **Munimbazi and Bullerman** did not identify the antifungal agent. In this study, five cyclic dipeptides were isolated from cell-free, culture supernatant of *Bacillus amyloliquefaciens* subsp. plantarum strain FZB42. The five peptides were identified as: *cis*-Cyclo(L-Pro-L-Pro) (1), cis-Cyclo(L-Pro-L-Val) (2), *cis*-Cyclo(L-Pro-L-Ile) (3), *cis*-Cyclo(L-Pro-L-Leu) (4) and *cis*-Cyclo(L-Pro-L-Phe) (5). This report that cyclic peptide is responsible for antifungal activity the bacterial isolate is supported by **Magnusson et al. (2003)**; the group reported that the antifungal activity of *Lactobacillus coryniformis* strain Si3 could be due to Cyclo(phe-Pro) and Cyclo(Phe-4-OH-Pro) that were identified from bacterial culture supernatant. While one of these five peptides, *cis*-Cyclo(L-Pro-L-Val) (2) in the current study was previously reported to have an antifungal effect, the roles of the other four metabolites are not exactly known. Purified antifungal peptides could be a better alternative to the use of whole bacterial cells as inhibitors of fungal phytopathogens. The biological active, antifungal peptide could also find uses in the food industries as preservatives, in cell culture and in treating human dermatophytes.

CONCLUSION

It can be concluded that this study supports previous findings that members of the genus *Bacillus* produce antifungal agents. The bacterial isolate that was identified as *Bacillus amyloliquefaciens* subspecies plantarum strain FZB42 produces five peptide metabolites; at least one of these peptide metabolites (cis-Cyclo(L-Pro-L-Val) is a known and unique antifungal agent that is resistant to high temperature and hydrolytic enzymes. Future work is to synthesize: cis-Cyclo(L-Pro-L-Pro) (1), cis-Cyclo(L-Pro-L-Ile) (3), cis-Cyclo(L-Pro-L-Leu) (4) and cis-Cyclo(L-Pro-L-Phe) (5) and study their efficacy against fungi and bacteria.

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ENHANCED AMYLOLYTIC ACTIVITY OF INTRACELLULAR α-AMYLASE PRODUCED BY BACILLUS TEQUILENSIS

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ABSTRACT

The amylolytic enzyme plays a very important role in industrial applications. This study aimed to screen amylase producing *Bacillus* sp. and to promote its amylolytic activity by mutagenesis. Samples were collected from coastal mud samples and starch hydrolyzing isolates were screened. A single isolate having the highest enzyme activity was identified as *Bacillus tequilensis* by 16S rRNA analysis. A starch medium was optimized and fermentation period studies revealed that the mutant strain (after 60 sec of UV exposure) had higher activity (868 U/mL/min) than the parental strain (418 U/mL/min) after 36 hours of incubation at 37°C, pH 7.0. It was also found that amylase from intracellular mutant strain had maximum activity; on the other side parental strain had maximum activity with an extracellular enzyme. Optimized temperature, pH and salt concentration revealed that the intracellular amylase from mutant strain had the maximum activity of 978 U/mL/min, 985 U/mL/min, 960 U/mL/min respectively. Varying the source of carbon in the medium had a significant impact on enzyme activity. Metalloenzymes like amylases were reported to have strong activity towards metal ions, so amylase activity was analysed by adding different metal ions in the medium and found that calcium ions strongly promoted amylase activity and Fe^{2+}, Zn^{2+}, Cu^{2+}, Mg^{2+} inhibited the activity. SDS-PAGE results showed that the molecular weight of isolated amylase to be approximately 55.0 kDa. Our study showed the capability of mutant *B. tequilensis* strain to produce double the amount of intracellular amylase than the parental strain.

Keywords: Amylolytic, mutagenesis, metalloenzymes, metal ions, zymogram

INTRODUCTION

Amylases are extracellular enzymes which hydrolyze the alpha 1, 4 glycosidic linkages of starch into sugars. Based on the cleavage site, amylase was classified into three types namely α- amylase, β- amylase and γ- amylase. Among them, α-amylase is one of the significant industrial enzymes which are widely utilized for the purpose of brewing, baking, textile and detergent (**Gupta *et al.*, 2003**). The biologically active enzymes are immensely present in natural resources such as plants, animals and microorganisms (**Mageswari *et al.*, 2012**) of which microbial source plays a vital role since it can be produced in huge quantity meeting the demands of the market (**Pandey *et al.*, 2000**). *Bacillus tequilensis* is a Gram-positive, motile rods, produces central endospore in unswollen sporangia and its pathogenicity was undetermined (**Gatson *et al.*, 2006**). Several studies on ultraviolet (UV) light have been reported to be mutagenic in a variety of organisms. The impact of UV radiations on alpha amylase producing ability of *Bacillus* species were studied earlier and attempts have been made for the overproduction of microbial enzyme by induced mutagenesis (**Demirkan, 2011**). The increase in amylase activity after exposing to UV light may be due to the changes in the promoter zone of a gene encoding for this enzyme and also the transcription of mRNA corresponding to the enzyme might have deregulated (**De Nicolas-Santiago *et al.*, 2006**). The impact of ultraviolet radiations on *Bacillus tequilensis* producing α-amylase was studied for the first time and the comparison of mutant and wild (parental) strain for increased α-amylase activity was performed.

MATERIALS AND METHODS

Sample collection

Samples were collected from the coastal environment in Pudukkottai district, (10° 1' 12" North, 79° 13' 35" East) Tamil Nadu, India. Under sterile conditions, mud samples were collected and the samples were transferred to the laboratory and stored at -20°C until further processing. The samples were collected from three different spots; [I] from the top layer (PDU-1), [II] from 10cm depths (PDU-2) and [III] near the trees (PDU-3).

Isolation, screening and identification of α-amylase producer

Collected samples were used for the isolation of amylase producing bacteria. Samples were processed by taking one gram of mud sample suspended in 9.0 mL of sterile water and agitated for a min. 0.1 mL of the suspended sample was spread plated onto nutrient starch agar (2.0% starch, 0.75% peptone, 0.5% beef extract, 0.5% NaCl, 1.8% agar) and incubated at 37°C for 24 hours. To identify the amylolytic bacteria, plates were overlaid with iodine reagent (0.01 M I_2-KI). Based on the starch hydrolysis properties, isolates were identified by the clear zone formed around the isolates. All the isolates that produced a clear zone of hydrolysis (Amy+ and Amy-) were further selected and screened for amylase production. Selected positive isolates (Amy+) were sub-cultured twice for purity and pre-cultures were maintained in nutrient agar slants (-20°C). Single selected isolate B- PDU2/2 was further identified by 16S rRNA sequencing. DNA was isolated using Hi-media bacterial DNA mini-prep kit as per manufacturer's protocol and amplified using universal 16S rRNA primers; 27F: 5'-AGAGTTTGATC MTGGCTCAG- 3' and 1492R: 5' - CGGTTACCTTGTTACGACTT- 3' (**Prasanth *et al.*, 2016**). Species level identification was done at www.ncbi.nlm.nih.gov/BLAST/ and BLAST nucleotide sequence similarity with 98% or above was deemed as sufficient for species identification.

Inoculum and crude enzyme preparation

Inoculum preparation was done by inoculating overnight grown culture from starch agar plates into 50 mL of starch broth and incubated at 37°C for 24 hours.

Exponential phase was achieved that contained $3.0X10^8$ CFU/mL and suitable volume from this suspension was used for the tests. To prepare crude enzymes, inoculated overnight grown cultures in starch broth were centrifuged at 10,000 rpm for 15 min and the supernatant was used as a crude enzyme for the enzyme assay for determination of activity.

Enzyme assay

The enzyme activity was assayed by using the DNS method. Briefly, a reaction mixture contained 0.5 mL crude enzyme solution and 0.5 mL soluble starch (1 g/100 mL- prepared using 0.01M phosphate buffer) that was incubated for 5 min and reducing sugar was measured. The reaction mixture without crude enzyme served as a control (**Miller, 1959**). One unit of amylase activity is defined as the amount of enzyme that produces one micromole of reducing sugar in one minute at a constant temperature using soluble starch as substrate. Lowry's protein estimation method was used for total protein content determination (**Lowry *et al.*, 1951**).

Mutagenic studies

The grown overnight bacterial culture was centrifuged at 10,000 rpm for 15 min and the bacterial cells were resuspended in 50 millilitres of peptone water and further diluted to 10^6 times and dilutions were plated, that served as a control. Ten millilitres from the diluted culture was transferred to another sterile petri plate. Mutagenesis by UV rays was studied by exposing the petri plates at different time intervals (30s, 60s, 90s and 120s) under the UV lamp. After exposing to pre-determined time intervals, 0.5 mL of bacterial culture was plated onto a starch agar plate and incubated at 37°C for 24-48 hrs and further used for enzyme assay. The intracellular and extracellular activity of amylase was studied for both parental and mutant strains. To extract the intracellular amylase, cells were sonicated and centrifuged to collect the intracellular crude enzyme.

Optimization of physiochemical and nutritional parameters

Parameters that can influence the amylase production were optimized independently and individually. So, optimization conditions were used subsequently for all the experiments both for the parental and mutant strain. For temperature optimization, the basal medium was inoculated and incubated at 35, 40, 45, 50, 55°C under standard assay conditions and for every 12 hours samples were withdrawn to study the effect of the incubation period. For pH optimization, the culture medium was prepared with variable pH 5.0 to 9.0 and assayed for standard assay conditions. The growth medium was supplemented with varying concentrations of starch (1% to 4%) and also with different carbon sources including maltose, sucrose, lactose, glucose and fructose for enzyme production. To analyse the effect of salt concentration on enzyme production varying concentrations of sodium chloride (1% to 6%) was used in the growth medium.

Effect of metal ions on enzyme activity

Metal ions have a strong influence on enzyme production and activity. To investigate the role of metal ions towards amylase activity various metal ions, $FeSO_4$, KCl, $CuSO_4$, $ZnSO_4$, $CaCl_2$, $MgSO_4$ were used at 25 mM concentration. Different metal ions were added to the medium and enzyme activity was assayed in standard assay conditions (**Demirkan, 2011**).

SDS-PAGE and zymogram analysis

The molecular weight of the enzyme was estimated by sodium dodecyl sulphate polyacrylamide gel electrophoresis. The prepared enzyme from both parental and mutant strain was loaded along with protein marker in 12% gel and purity of the protein was confirmed (**Laemmli, 1970**). Approximate molecular weight was determined after staining with CBB and de-staining. Zymogram was done with native-PAGE by incubating in 1% starch for 1 hour and staining with iodine solution.

Kinetic properties of α-amylase and statistical analysis

To assess the kinetic properties, hydrolysis by DNS method with different concentrations of starch was used. K_m and V_{max} were calculated using the Michaelis-Menten equation. All the experiments were performed in triplets and the resulting values were presented as the mean of three independent observations. GraphPad Prism 5.0 software was used for calculating mean-standard deviation for each experimental result.

RESULTS AND DISCUSSION

Isolation, screening and identification of amylase producing bacterial cultures

Bacterial isolates producing clear amylolytic zones were initially identified by staining the plates with iodine solution. 16 isolates were found to produce clear zones in the starch-iodine plate out of which six isolates produced >1.7 cm zones that are considered as significant. Selected isolates were reassessed for the zone of clearance by a well-diffusion method. Isolates that showed the constant zone of clearance within 5-6 hours of incubation at 37°C was selected for further analysis. Isolate B-PDU2/2 was selected and found to be Gram-positive, motile and facultative aerobe. It was found to grow at pH 6.0, 7.0, 8.0 and salt concentrations up to 7%. Analysis of 16S rRNA sequence revealed its 98% homology with *Bacillus tequilensis* (KT760402). 16S rRNA is a powerful molecular marker for species-specific identification at the microscopic level was provided earlier (**Tiwari *et al.*, 2014**).

Medium selection and mutagenic experiments

Growth curve experiments revealed that B-PDU2/2 had a steady stationary phase after 30 hours in starch broth while other nutrient broths had 24 hours. Extracellular amylase activity was found to have maximum activity/productivity at 36 hours in starch broth (Fig.2). Mutagenic studies with UV exposure at different time intervals revealed that after 60 secs of UV exposure maximum amylase activity was achieved in the intracellular enzyme (Fig.1). Interestingly, the parental strain had a maximum activity of 418 U/mL/min with extracellular enzyme whereas intracellular enzyme from mutant strain had a maximum activity of 868 U/mL/min. Hence, the starch broth with the extracellular enzyme from parental strain and intracellular enzyme from a mutant strain (60 secs of UV exposure) was further taken for optimization. Similarly, higher activity was observed in the mutant strain than in its parental strain in *B. subtilis* (**Zhao and Qirong, 1994; Allan *et al.*, 1997**). In some cases, UV mutagenesis also inhibited the activity of parental strain that showed lower activity than parental strain in contrast chemical mutagenesis had higher activity (**Haq *et al.*, 1997**).

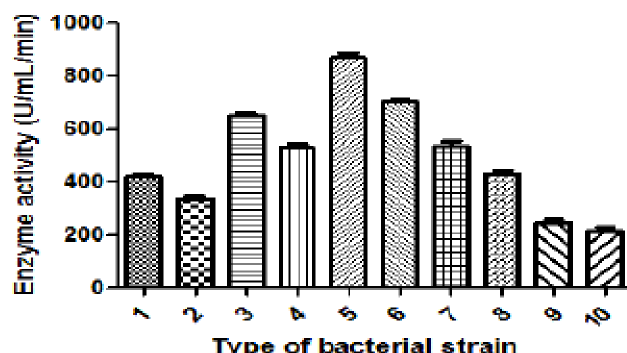

Figure 1 Comparison of amylase produced by parental and wild type strains of *Bacillus tequilensis* in starch medium at 45°C, pH 7. 1-parental extracellular, 2-parental intracellular, 3-after 30s of UV exposure intracellular, 4-after 30s of UV exposure extracellular, 5-after 60s of UV exposure intracellular, 6-after 60s of UV exposure extracellular, 7-after 90s of UV exposure intracellular, 8-after 90s of UV exposure extracellular, 9-after 120s of UV exposure intracellular, 10-after 120s of UV exposure extracellular.

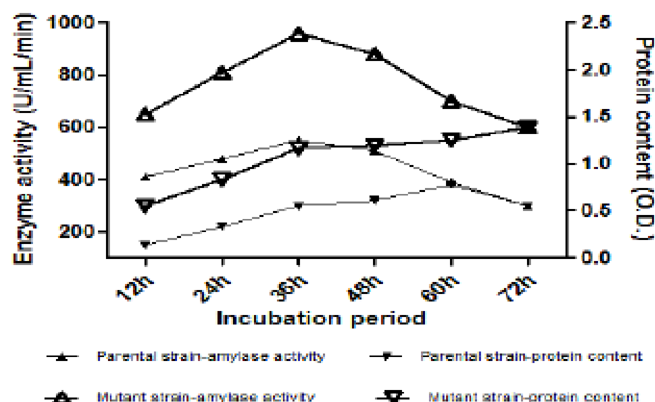

Figure 2 Total protein content of *Bacillus tequilensis* compared with production of amylase in mutant (60 sec UV exposure, intracellular) and parental strain (extracellular). Fermentation period analysis was made in starch medium at 45°C, pH 7.0.

Effect of different temperature

Evaluation of amylase activity at different temperatures (35-65°C) showed that both parental extracellular and mutant intracellular enzyme had a maximum activity of 522 and 978 U/mL/min at 45°C respectively. It also showed that

increase in temperature gradually decreases the activity of amylase (Fig.3,4). The temperature was also found to have a profound effect on *Bacillus* sp. in amylase production (**Raul *et al.*, 2014**). An incubation temperature of 37°C was optimum for most *Bacillus* sp. to produce maximum activity of amylase though some thermostable amylase can sustain temperatures up to 45 – 90°C (**Sodhi *et al.*, 2005; Asgher *et al.*, 2007**).

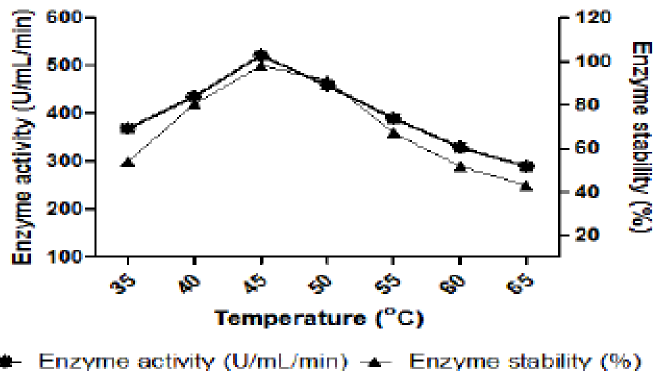

Figure 3 Effect of different temperature on enzyme activity and enzyme stability of parental strain (extracellular). Each individual point is the representation of mean with standard deviation for each experimental result done in triplicates.

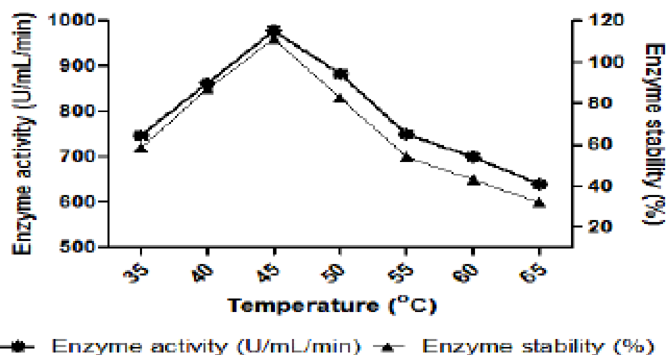

Figure 4 Effect of different temperature on enzyme activity and enzyme stability of mutant strain (60 sec of UV exposure, intracellular). Each individual point is the representation of mean with standard deviation for each experimental result done in triplicates. Mutant strain was found to have more enzyme activity as well as production rate comparing with parental strain.

Effect of different pH

Optimization with different pH (5.0 – 9.0) revealed that both extracellular and intracellular amylase were not stable at stronger acidic as well as alkaline conditions. Also, pH of the medium had a strong influence on enzyme activity (Fig.5,6). At neutral pH 7.0 extracellular amylase of parental strain had an activity of 538 U/mL/min and intracellular amylase from the mutant strain (985 U/mL/min) had maximum activities. *Bacillus* sp. used for industrial amylase production was found to have optimum pH of 6.0 to 7.0 (**Haq *et al.*, 2010**). Variation in pH also indicates the initiation and end of enzyme synthesis (**Friedrich *et al.*, 1989**). The incubation period of 36 hours was found to have maximum enzyme activity (fig.2) and increase in incubation time decreases the enzyme activity that may be due to an interaction of synthesized enzyme with other components in the medium (**Ramesh and Lonsane, 1987**).

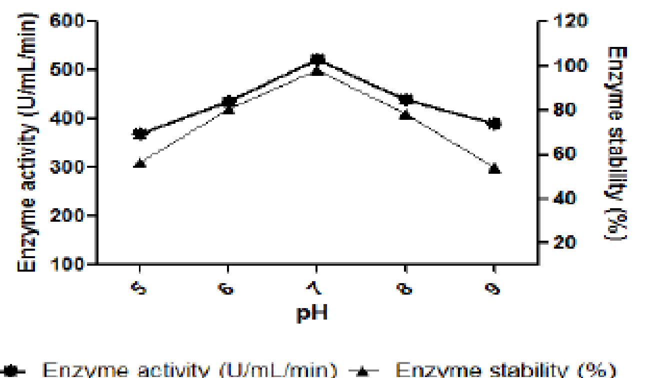

Figure 5 Effect of different pH on enzyme activity and enzyme stability of parental strain (extracellular). Each individual point is the representation of mean with standard deviation for each experimental result done in triplicates.

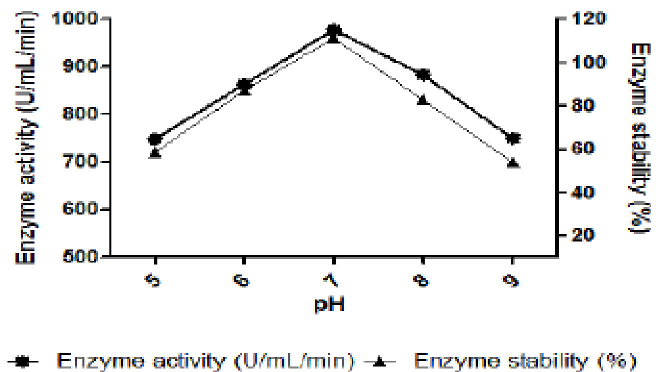

Figure 6 Effect of different pH on enzyme activity and enzyme stability of mutant strain (60 sec of UV exposure, intracellular). Each individual point is the representation of mean with standard deviation for each experimental result done in triplicates. Mutant strain was found to have more enzyme activity as well as production rate compared to parental strain.

Effect of salt concentration on enzyme production

Enzyme activity was found to be decreasing as salt concentration (NaCl) increases from 1%. Though bacterial growth was observed at higher salt concentrations up to 7% enzyme activity was inhibited with an increase in salt concentration (Fig.7). Accordingly, the parental strain had maximum activity at 1% (510 U/mL/min) as same as the mutant strain (960 U/mL/min).

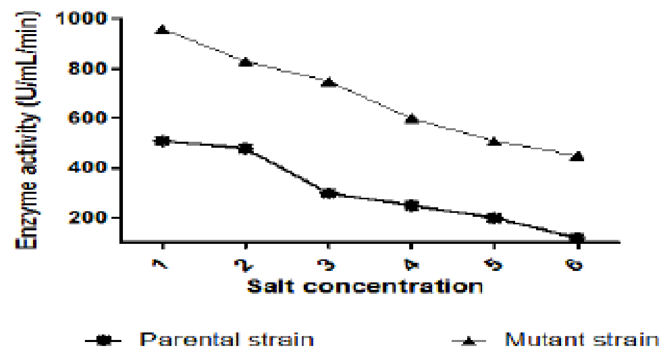

Figure 7 Effect of different salt concentration (NaCl) on enzyme activity of parental strain (extracellular) and mutant strain (60 sec of UV exposure, intracellular). Each individual point is the representation of mean with standard deviation for each experimental result done in triplicates.

Effect of different carbon sources

The starch broth was found to give maximum enzyme activity/production. Any increase or decrease in starch concentration (1% to 4%) had a lesser effect on enzyme activity on both parental and mutant strains (510 U/mL/min and 960 U/ml/min) whereas 2% starch had maximum activity after 36 hours of incubation (Fig.8). Supplemented with other carbon sources had affected the enzyme activity in that case glucose and fructose gave lesser enzyme activity than maltose, sucrose and lactose (maltose>lactose>sucrose>glucose>fructose). Some reports found maltose to be a good inducer of amylase activity (**Goto *et al.*, 1988; Narang and Satyanarayana, 2001**). In some cases, glucose was found to repress amylase activity and also glucose was reported to be an inducer (**Normurodova *et al.*, 2007**). Varying starch concentration was also reported influencing the enzyme activity (**Tiwari *et al.*, 2014**).

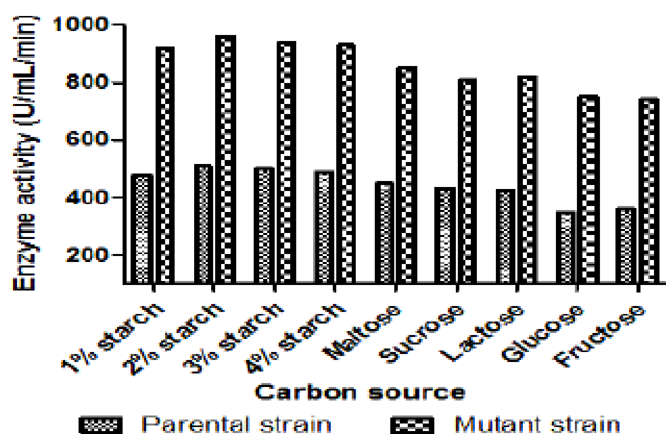

Figure 8 Effect of different carbon sources on enzyme activity of parental strain (extracellular) and mutant strain (60 sec of UV exposure, intracellular). Each individual bars are the representation of mean with standard deviation for each experimental result done in triplicates.

Effect of different metal ions

For the study of metal ions, 25mM each of $FeSO_4$, KCl, $ZnSO_4$, $CaCl_2$, $CuSO_4$, $MgSO_4$ were used in the medium at optimum temperature, pH and incubation period. Out of which calcium ions was found to be best for amylase production. Other ions Fe^{2+}, Zn^{2+}, Cu^{2+}, Mg^{2+} had slightly decreased the enzyme activity compared to Ca^{2+} ions (Fig.9). It was found that calcium ions increase the activity of amylase compared to control (without ions). The catalytic activity of amylolytic enzymes can be affected by mono- and divalent metal ions but this metalloenzyme has up to six Ca^{2+} atoms at its active site so that can be activated by calcium ions (**Asgher *et al*., 2007; Normurodova *et al*., 2007**).

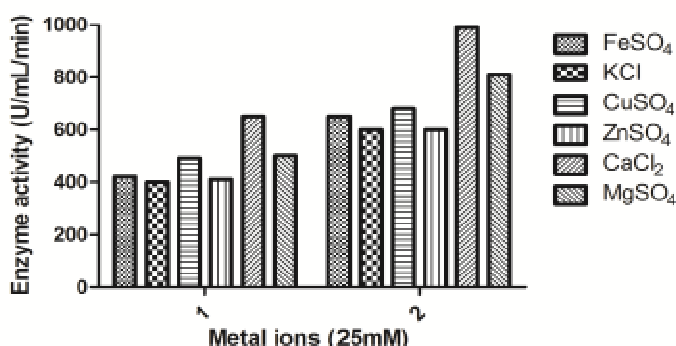

Figure 9 Effect of different metal ions on enzyme activity of parental strain (extracellular) and mutant strain (60 sec of UV exposure, intracellular). 1- Parental strain and 2- Mutant strain. Each individual bars are the representation of mean with standard deviation for each experimental result done in triplicates.

Stability of amylase and kinetic properties

Stability of enzyme was particularly important for its application in industries. Amylase produced from *B. tequilensis* was found to be active and stable at 45°C and pH 7.0 that correlates with its higher production rate (Fig.3,4,5,6). Protein content and amylase activity were found to be strongly influenced by the temperature and pH, although the temperature was controlled by incubation conditions and pH in the medium. Amylase from *Bacillus* spp. was also reported being stable at acidic as well as alkaline conditions (**Demirkan *et al*., 2005; Demirkan, 2011**). Michaelis-Menten kinetic parameters were calculated to be 1.27 mg/mL and 1.97 mg/mL for K_m and 121 U/mL and 187 U/mL for V_{max} respectively.

SDS-PAGE analysis and zymography

The molecular weight was determined to be approximately 55.0 kDa (Fig.10) and a single band in SDS-PAGE confirmed the purity of amylase produced from *B. tequilensis*. Alpha-amylase from *Bacillus* spp. was found to have a molecular weight ranging from 50-60 kDa. A similar study using *B. tequilensis* reported molecular weight to be 67 kDa for synthesized amylase (**Tiwari *et al*., 2014**). Zymogram results also indicated the presence of an amylolytic enzyme that hydrolysis the starch. The clear zone indicates the presence of an enzyme (protein) in zymography (Fig.11).

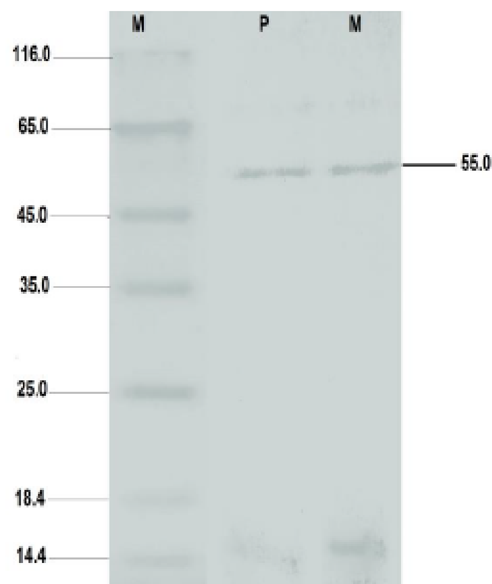

Figure 10 SDS-PAGE results for amylase from parental and mutant strains of *Bacillus tequilensis*. M- Protein marker, P-extracellular amylase from parental strain, M- intracellular amylase from mutant strain. Molecular weights were represented in kDa and produced amylase was ~55.0 kDa.

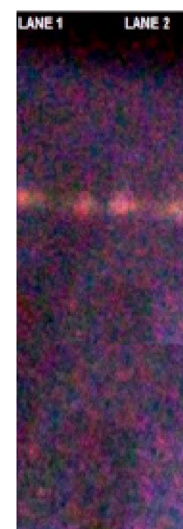

Figure 11 Zymogram result for amylase from *B. tequilensis* in native PAGE that hydrolysed soluble starch. A clear white region around the black background indicates the amylolytic activity. Lane-1; extracellular amylase from parental strain, Lane-2; intracellular amylase from mutant strain.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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IN VITRO CHARACTERIZATION OF LACTIC ACID BACTERIA ISOLATED FROM LASODA BARI- A RARE FERMENTED FOOD OF HIMACHAL PRADESH-INDIA FOR POTENTIAL PROBIOTIC ATTRIBUTES

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ABSTRACT

Probiotic lactic acid bacteria (LAB) are health promoting microorganisms which are recently been used as food additives and therapeutic supplements. Recently, there has been an increase of interest regarding the commercial utilization of probiotic LAB strains isolated from traditional and naturally fermented food products. Therefore, the present study was aimed to isolate and screen lactic acid bacteria from Lasoda bari – a rare fermented food of Himachal Pradesh, for the first time for their probiotic potential. Total eight isolates were obtained out of which one isolate was selected based on its broadest antagonistic spectrum. This strain was identified using 16S rRNA technique as *Pediococcus pentosaceus* LB-CC. The analysis of acid resistance, bile tolerance, antibiotic sensitivity, auto-aggregation and microbial adhesion to organic solvents were established. The results revealed normal growth of *P. pentosaceus* LB-CC in the presence of low pH, high bile salt concentration and ability to produce antimicrobial compounds. No gelatinase and DNase enzyme activity was detected. Natural susceptibility to the tested antibiotics was observed. Thus, according to these results, this probiotic strain could be proposed as safe potential probiotic culture and can be exploited for the production of nutraceutical agents.

Keywords: Probiotics, lasoda bari, lactic acid bacteria, *Pediococcus*, Himachal Pradesh, safety

INTRODUCTION

Health-promoting bacteria commonly referred to as probiotics, have been shown to improve the intestinal microbial balance and the properties of the indigenous microflora (**Mattila-Sandholm *et al.*,1999**). Probiotics are defined as 'Live microorganisms which when administered in adequate amounts confer a health benefit on host' (**FAO/WHO, 2002**). The term probiotics refers to viable, non-pathogenic microorganisms (bacteria or yeasts) that, when ingested, are able to reach the intestine in sufficient numbers to deliver health benefits to the host (**Hawaz, 2014**). Lactic acid bacteria (LAB) are one of the groups of microorganisms that dominate fermented foods and are potential probiotic candidates (**Guasch-Jane *et al.*, 2005**). LABs are widely used as in a variety of food preparations including dairy, meat and vegetables (**Silva *et al.*, 2013**). The species belonging to the group of LAB like *Lactobacillus, Lactococcus, Pediococcus, Streptococcus,* etc. and genus *Bifidobacterium* are the most commonly used probiotics (**Soccol *et al.*, 2011; Saad *et al.*, 2013**). *Pediococcus* are gram positive, coccus, being able to colonize the digestive tract and can prevent cardiovascular diseases, prevent harmful pathogens from accessing the gastrointestinal mucosa and provoke immune reactions (**Nghe and Nguyen, 2014**).
Isolation and screening of lactic acid bacteria from naturally fermented rare and novel food products have always been the most powerful means for obtaining useful cultures for scientific and commercial purposes. The proper selection and balance of lactic acid bacteria used for starter culture is critical for the manufacture of fermented food products with their desirable texture and flavor (**Sanders, 2000**). LAB are commonly used in most probiotics preparations due to being the desirable members of the intestinal microflora and thus beneficially amend the balance of intestinal microflora, inhibit the growth of harmful bacteria, promote good digestion, boost immune function and increase resistance to infection (**Ahn *et al.*, 2002**). A potential probiotic bacterium must qualify certain selection criteria such as acid and bile stability, antimicrobial production and antagonistic activity and should be preferably of human origin.
Currently, traditional fermented products are receiving new attention for their health promoting and disease preventing/curing effects, i.e., probiotic significance. These probiotic strains are acid tolerant and may be adaptable to intestinal conditions and survive the passage through the gastrointestinal tract. This opens up the possibility to use potential probiotic strains from fermented food products other than those of animal origin that are commonly used (**Lindstrom *et al.*, 2012**). The main objectives of this study were to screen novel LAB from Lasoda bari – a rare and traditional fermented food product of Himachal Pradesh for its safety by antibiotic susceptibility test, hemolysis, DNase and gelatinase enzyme production ability and assessment of probiotic qualities that include acid-bile tolerance, auto-aggregation, hydrophobicity and antagonistic potential.

MATERIAL AND METHODS

Isolation of Lactic acid bacteria

LAB strain was isolated from Lasoda bari - a rare and traditional fermented food product of Himachal Pradesh (shown in figure 1) using De Man Rogosa and Sharpe (MRS) broth (**De Man *et al.*, 1960**) by serial dilutions method and incubated at 35 °C for 24-48 h anaerobically. Lasoda bari is traditionally prepared from Lasoda (*Cordia dichotoma*) and black gram (*Vigna mungo*). Lasoda bari prepared from Lasoda fruits, being rich many medicinal properties viz. anti-inflammatory, remedying the effects of an inflamed colon and liver, diuretic, anti-ulcer and antidiabetic properties have been explored for the first time to isolate rare and potential probiotic strains. Pure strains, as judged by microscopic observations for homogeneity of cellular morphology, were maintained in 30 % glycerol at 4 °C. In total 8 isolates were obtained and were further tested for Gram reaction, catalase test, cell morphology and antimicrobial activity. Isolates LB-CC was selected for further study on the basis of its antagonistic spectrum against spoilage and food borne pathogens by using Bit/disc method. On the basis of 16S rRNA gene technique LB-CC was identified as *Pediococcus pentosaceus*. The sequences so obtained were submitted in National Center for Biotechnology Information (NCBI) to get an accession number. *P. pentosaceus* LB-CC registered under the accession number KM251460.

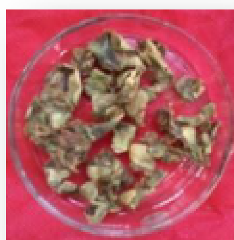

Figure 1 Lasoda bari

Safety assessment of LAB

One of the most important criteria for bacterial strains intended for use in the food industry is concern for their safety.

Antibiotic susceptibility

The antibiotic susceptibility was determined towards antibiotics viz. Ampicillin, Augmentin, Gentamycin, Cephalosporin, Cloxacillin, Cefotaxime, Cefoxitin, Lincomycin, Tetracyclin, Amoxyclave, Co-trimoxazole and Cefuroxime. Antibiotic–impregnated discs (Hi-media, India) were placed on seeded plates and the zone of growth inhibition was observed after 24h of incubation at 35°C to detect their susceptibility for antibiotics.

Hemolytic activity

Hemolytic activity of selected isolate was determined by spot inoculating fresh overnight bacterial culture on Blood Agar plates (HiMedia) and incubated at 35 °C for 24-48 h (**Linaje *et al*., 2004**).

DNase production

DNase enzyme production of probiotic isolate was evaluated by following **Gupta and Malik (2007)**. A clear pinkish zone around the colonies against dark blue background was considered as positive result for DNase enzyme production.

Gelatinase production

Gelatinase enzyme production of isolate was determined by streaking 24 h old culture on plates containing MRS agar supplemented with 3 % gelatin. Development of clear zones around the colony against the opaque background indicated a positive reaction (**Harrigan and McCance, 1990**).

Assessment of probiotic attributes:

Tolerance to Low Acid conditions

To evaluate the low pH tolerance ability of the culture the method of **Liong and Shah (2004)** was followed with slight modifications. Buffers of different pH viz. 1, 2, 3 and 6.5 were used to evaluate the tolerance of selected isolate for low pH for 3 h. Acid tolerance was determined by comparing the final plate count after 3h with the initial plate count at 0 h.

Effect of bile salts on the growth rate of isolates

Effect of bile on the growth of selected isolates was studied by the method **Giililand and Walker (1990)**. Viability of cells in MRS broth supplemented with 0.3, 1 and 2 % bile salts upto 8 h was observed by plating 100 µl of culture onto MRS agar plates and incubated at 35 °C for 24 h. Growth of bacteria was expressed in colony forming units per milliliter (log CFU/ml) and the percent survival of strain was then calculated.

Survival in simulated *in vitro* digestion

Survival in simulated gastric and intestinal juice was determined following the method given by **Charteris *et al.* (1998)**. The pH in human stomach ranges from 1, during fasting, to 4.5 after a meal, and food ingestion can take up to 3-4 h. Thus, the tolerance was assayed by determining the viable count in simulated gastric juice after the incubation for different time intervals up to 4 h. All the experiments were carried out in triplicates.

Auto-aggregation

Auto-aggregation assay was performed as described by **Del Re *et al.* (2000)**. Optical density (OD) of bacterial cells LB-CC suspended in phosphate buffer saline (PBS) was set to 0.5 at 600 nm followed by incubation at 35 °C for 5 h. Auto-aggregation % was measured as 1- (At/A0) × 100, where At represents the absorbance at time t = 1, 2, 3, 4, 5 h and A0 the absorbance at t = 0 h (i.e. 0.5)

Co-aggregation

Co-aggregation ability of selected isolated was determined by following the method described by **Del Re *et al.* (2000)**. Mixtures were made for the selected isolate with pathogenic bacteria viz. *Listeria monocytogenes* MTCC 839, *Clostridium perfringens* MTCC 1739 and *Bacillus cereus* CRI at 1:1 ratio. Probiotic bacterial cells and indicator bacteria were kept as control and were incubated at 35 °C for 4 h. Absorbance at λ = 600 nm was observed for mixture and each of individual strain. Co-aggregation % was calculated according to Handley's equation (**Handley *et al.*, 1987**).

Antimicrobial activity

Antimicrobial activity of cell free supernatant of isolate was checked against *Listeria monocytogenes* MTCC 839, *Leuconostoc mesenteroides* MTCC 107, *Enterococcus faecalis* MTCC 2729, *Bacillus cereus* CRI, *Clostridium perfringens* MTCC 1739, *Pectobacterium carotovorum* MTCC 1428, *Escherichia coli* IGMC, *Pseudomonas syringae* IGMC and *Staphylococcus aureus* IGMC. The well with the holding volume of 150 µL was made in the center of the plate using well cutter. Samples of 24 h old cultures of isolate were centrifuged at 12,000 x g for 10 min, and 150 µL of the supernatant was loaded in the well and the plates were incubated at 35 °C for 24 h (**Kimura *et al.*, 1998**). The antibacterial activity was determined and zones of inhibition were measured in millimeter (mm).

H_2O_2 production

Quantitative estimation of Hydrogen Peroxide (H_2O_2) was done by following the method given in **AOAC (1995)**.

HPLC- determination of lactic acid

As *Pediococcus* are homo-fermentative bacteria, major end product of their metabolism is lactic acid. Lactic acid production by *P. pentosaceus* LB-CC was detected by using HPLC (Novapak C-18) column, 490E multiwavelength UV detector, Millennium 2010 data processor and Rheodyne injector with 20 µl loop. Mobile phase used was Methanol : Water (double distilled) (95 : 5). Standard organic acid solution i.e. 5 % of lactic acid (Sigma Aldrich) was prepared in double distilled water. HPLC analysis was firstly performed with standard organic solution followed by the samples. The monitoring was done at 210 nm.

RESULTS AND DISCUSSION

Isolation and biochemical characterization

Eight Lactic acid bacteria isolates were obtained from Lasoda bari and 6 out of 8 were confirmed as rods while 2 were confirmed as coccus (tetrad) as revealed by microscopic examination, were non sporulating, catalase negative, not able to utilize citrate, no casein hydrolysis, no urease production and no indole production were observed. On the basis of microscopic examination isolates were tentatively identified as *Lactobacillus* and *Pediococcus* sp. Out of eight isolates, LB-CC gave clear halos around the indicator pathogenic strains using bit/disc method with widest antimicrobial spectrum and was selected for further study.

The largest diameter of inhibition upto 23.6 mm was obtained against serious food borne and spoilage pathogens viz. *L. monocytogenes*, *S. aureus* and *C. perfringens*, revealing their antagonistic potential and use as safe biopreservative. Similar antagonistic pattern was observed by **Nghe and Nguyen (2014)** where *P. pentosaceus* VTCC-B-601 showed effective antimicrobial effect against serious food borne pathogens *Staphylococus aureus* ATCC 25923, *Salmonella typhimurium* ATCC 19430, *Pseudomonas aeruginosa* ATCC 27853 and *Micrococcus luteus* ATCC 10240. **Hawaz (2014)** also studies the antimicrobial activity of *Lactobacillus* isolates against pathogentic bacteria viz. *Staphylococcus* sp., *Bacillus* sp., *Pseudomonas* sp. and *E. coli* and found them to show in vitro inhibitory zones.

Analysis of the 16S rRNA sequences revealed that lactic acid bacteria isolated from Lasoda bari displayed 99% homology with *Pediococcus pentoseceus* DSM 20336 as shown in figure 2. The 16S rRNA gene sequences were deposited in gene bank under accession no. KM251460 for *Pediococcus pentoseceus* LB-CC. Number of nodes in neighbor- joining phylogenetic tree are levels of bootstrap support (%) from 1000 resample database. The isolate has been reported for the very first time from Lasoda bari- a rare and novel fermented food product of Himachal Pradesh with a very good probiotic potential.

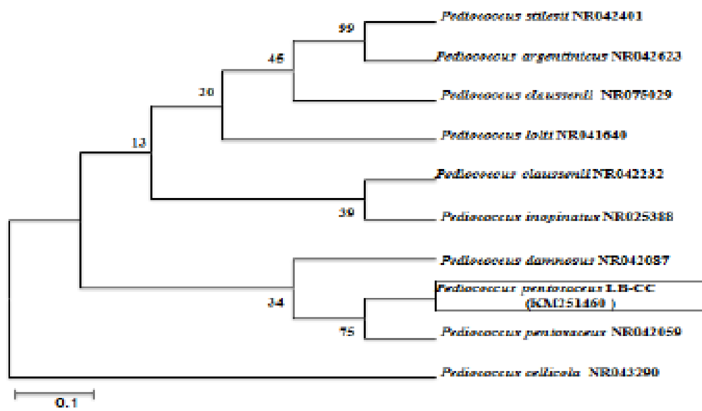

Figure 2 Neighbor joining phylogenetic tree of *Pediococcus pentosaceus* LB-CC based on 16S rRNA gene sequence using Mega 6

Table 1 Antibiotic sensitivity of *P. pentosaceus* LB-CC

S. No	Antibiotics	Concentration (µg)	*S/R
1.	Ampicillin (AMP)	30	S
2.	Augmentin (AMC)	30	S
3.	Gentamicin (GEN)	10	S
4.	Cephalothin (CEP)	30	S
5.	Cloxacillin (COX)	1	S
6.	Cefotaxime (CTX)	30	S
7.	Cefoxitin (CX)	30	S
8.	Lincomycin (L)	2	S
9.	Tetracycline (TE)	30	S
10.	Amoxyclav (AMC)	30	S
11.	Co-trimoxazole (COT)	25	R
12.	Cefuroxime (CXM)	30	S
% Sensitivity			91.66

Legend: S-Sensitive (Inhibited bacterial growth), R- Resistant (No effect on bacterial growth)

Safety assessment of isolates

P. pentosaceus LB-CC was found to be sensitive to all antibiotic used in the study (Tab 1), thereby presenting its inability to show resistance in the presence of antibiotics and thus their safe status. Isolate showed a negative response in the production of DNase and gelatinase enzymes as pathogenicity factors. No clear zones around colonies were observed on blood agar medium. Haemolytic activity would break down the epithelial layer while the gelatinase activity would damage the mucoid lining. Absence of haemolytic and gelatinase activity is a selection criterion for probiotic strains, indicating that these bacteria are non-virulent **(Marroki and Bousmaha-Marroki, 2014).**

Acid and bile tolerance

To be a successful probiotic, a bacterial strain must resist harsh conditions in stomach and gut region and must be able to colonize intestinal epithelium for its probiotic action. In this study we were able to obtain isolate that was able to grow even at pH 1 (during fasting) (Tab 2). *P. pentosaceus* LB-CC tested for survival in acidic environment at varied pH levels showed ability to grow well even at the minimum tested pH of 1.0 for 60 and 120 min of incubation, respectively. Since the lactic acid bacteria produce lactic acid during their fermentative metabolism, it is a known fact that they would be able to survive in gut's acidic environment. However, the acidic pH inside the gut would be from 2-4 in normal conditions (during fasting it may reach up to pH 1), the organisms that could effectively survive the lowest possible pH are more preferred for use in the food preparations **(Subhashini, 2014).** Therefore, in the present study, tolerance to low pH by *P. pentosaceus* LB-CC revealed its survival best under acidic conditions.

Table 2 Acid tolerance of *P. pentosaceus* LB-CC

pH	Incubation time (min)								
	Cell survival (log CFU/ml)*					**% Cell Survival			
	0	60	120	180	Mean	60	120	180	Mean
1.0	9.90	7.30	0.00	0.00	4.30	71.77 (57.88)#	0.00 (0.00)	0.0 (0.00)	**23.92 (19.29)**
2.0	9.90	7.60	7.30	7.00	**7.95**	74.47 (59.63)	71.50 (57.71)	68.2 (55.65)	**71.39 (57.66)**
3.0	10.00	10.05	9.80	9.60	**9.86**	98.80 (83.71)	96.07 (78.53)	93.65 (75.37)	**96.17 (79.20)**
Control	10.14	10.17	10.20	10.25	**10.19**	100 (89.96)	100 (89.96)	100 (89.96)	**100 (89.96)**
Mean	**9.98**	**8.78**	**6.82**	**6.71**		**86.26 (72.79)**	**66.89 (56.55)**	**65.46 (55.24)**	
$CD_{0.05}$	Treatment(T)= 0.495 Incubation Time (I)= 0.495 TxI= 0.991					Treatment (T)= 0.229 Incubation Time (I)= 0.198 TxI= 0.396			

*log CFU/ml: Mean of results from three separate experiments
**% Survivability = (log CFU/ml pH $_{1,2,3}$/ log CFU/ml pH$_{6.5}$) × 100
# Transformed values (Arcsign transformation)

The toxic effects of bile on bacterial cells are not well understood, but bile salts are surface-active, amphipathic molecules with a potent antimicrobial activity and they act as detergents that disrupt biological membranes **(Lebeer *et al.*, 2008).** The physiological concentration of bile salts in the small intestine is between 0.2- 2.0 % **(Gunn, 2000).** In this study, concentrations of 0.3, 1.0 and 2.0 % bile salts were used and effect of bile salt concentration on growth rate of isolate was studies. The culture when grown in 0.3, 1.0 and 2.0 % of bile salt concentration showed 95.04, 91.61 and 91.33 % survival on 8 h incubation as depicted in figure 3. The decrease in viable cells was observed when the concentration of bile salt was increased upto 2.0 %. It was considered that bile salt causes the increase in permeability of bacterial cell membranes, as the membranes are composed of lipids and fatty acids.

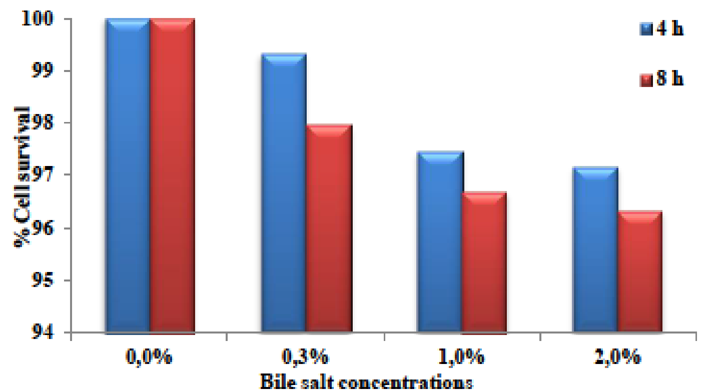

Figure 3 Bile salt tolerance of *P. pentosaceus* LB-CC

Tolerance to simulated gastric conditions

Gastrointestinal tract is the main location where the viability of lactic acid bacteria gets affected and the ability to survive in GI tract is one of the main desirable characteristics required for a probiotic. The survival of *P. pentosaceus* LB-CC at pH 2.0, 3.0 containing pepsin (depicting stomach conditions) and pH 8.0 containing pancreatin (depicting intestinal conditions) was observed for 4 h.

P. pentosaceus LB-CC exhibited good survival at pH 3 (5.99 log CFU/ml) upto 4 h and retained a moderate rate of survival at pH 2.0 (5.24 log CFU/ml) after 1 h of incubation (Tab 3). The results indicate that *P. pentosaceus* LB-CC may resist the effects of pepsin and pancreatin during the gastrointestinal (GI) transit therefore could be the potential source for probiotic formulations with effective delivery in GI tract.

Table 3 Percent survival of *P. pentosaceus* LB-CC in simulated gastric and intestinal juices

Gastro-intestinal juices	Incubation Time (h)						
	Cell survival (log CFU/ml)				Cell survival (%)		
	0	1	4	Mean	1	4	Mean
pH 2	9.8	5.2	0.0	**5.00**	49.61 (44.76)[#]	0.00 (0.00)	**24.80 (22.38)**
pH3	9.43	7.70	5.99	**7.70**	73.47 (58.97)	56.29 (48.59)	**64.88 (53.78)**
pH8	10.19	9.9	8.0	**9.36**	94.46 (76.35)	75.18 (60.09)	**84.82 (68.22)**
Control	10.25	10.48	10.64	**10.45**	100 (89.96)	100 (89.96)	**100 (89.96)**
Mean	**9.91**	**8.32**	**6.15**		**79.38 (67.51)**	**57.86 (49.66)**	
$CD_{0.05}$		Treatment (T)= 0.036 Incubation Time (I)= 0.032 TxI= 0.063				Treatment (T)= 0.005 Incubation Time (I)= 0.004 TxI= 0.007	

*Log cfu/ml: Mean of results from three separate experiments
**% Cell Survival = (log CFU/ml pH2,3,8/ log CFU/ml pH 6.5) × 100
[#] Transformed values (Arcsign transformation)

Auto-aggregation and Co-aggregation

Auto-aggregation was investigated on the basis of sedimentation characteristics and has been shown in figure 4. The sedimentation rate of isolates was measured over a period of 5 h. Results showed that *P. pentosaceus* LB-CC exhibited strong auto-aggregating ability (99 %). Better growth of the bacteria on MRS broth than on MRS agar could be the reason for slightly better auto-aggregation of cells grown on MRS broth. The observed auto-aggregation could be related to cell surface component, because it was not lost after washing and suspending of the cells in phosphate buffer saline (PBS) Kos ***et al.*, 2003).**

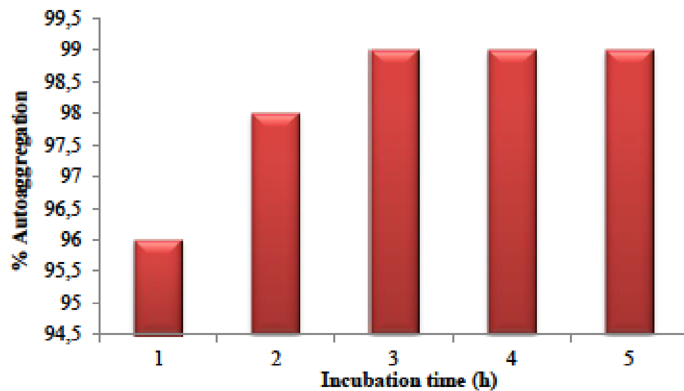

Figure 4 Auto-aggregation ability of *P. pentosaceus* LB-CC

Probiotic and pathogenic bacteria can combine together called co-aggregation. Probiotics are able to co-aggregate with pathogens and will efficiently inhibit and kill pathogenic bacteria as antimicrobial compounds can move directly on pathogens. *P. pentosaceus* LB-CC exhibited co-aggregative properties with all the pathogenic strains tested after 4 h incubation at 35 °C. The ability of *P. pentosaceus* LB-CC to co-aggregate with *B. cereus* (19.0 %) was significantly better than that with other two pathogenic strains viz. *L. monocytogenes* and *C. perfringens* (18.36 and 8.16 %,respectively) tested (figure 5).

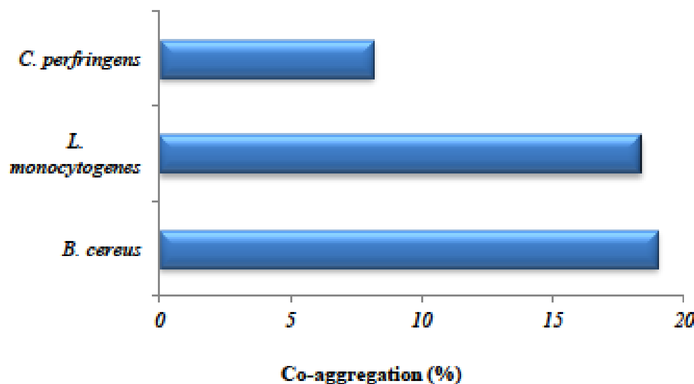

Figure 5 Co-aggregation ability of *P. pentosaceus* LB-CC with pathogens

Antimicrobial potential

Lactic acid production

P. pentosaceus LB-CC was able to inhibit the tested indicator organisms with varied zones of inhibition. The inhibitory activity of strains against pathogenic bacteria is shown in figure 6. Some species of *Staphylococcus aureus*, *Enetrococcus*, *Leuconostoc* and *Listeria* are highly pathogenic to human beings. The isolated *P. pentosaceus* LB-CC showed strong bactericidal activity against these species. Probiotic bacteria exhibited inhibitory zones against pathogenic bacteria in the range of 14.6 - 24.5 mm showing high inhibitory activity (>10mm) (Savagado ***et al.*, 2004). All this indicated a quite broad antagonistic spectrum of the strains.**

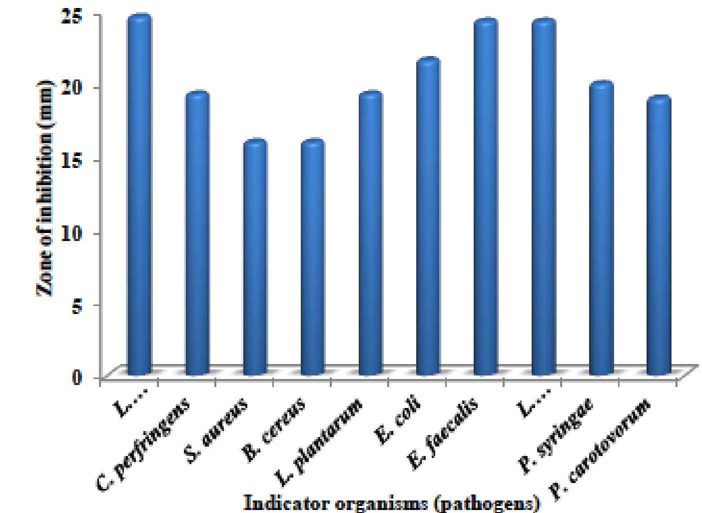

Figure 6 Antimicrobial activity of *P. pentosaceus* LB-CC against indicator strains at un-neutralized pH

The pH and titratable acids in the cell free culture supernatant of *P. pentosaceus* LB-CC and were 3.43 and 0.09 %, respectively. *Pediococcus* are homo-fermentative bacteria which produce more than 85% lactic acid from glucose being the major product of fermentation. *P. pentosaceus* LB-CC was homo-fermentative culture for lactic acid production which was quantified by HPLC system (Novapak C-18) and has been found to be 7.844 mg/L, after 24 h of incubation. Figure 7 shows typical HPLC chromatogram of standard and lactic acid extracted from culture supernatant of *P. pentosaceus* LB-CC revealing their antimicrobial potential.

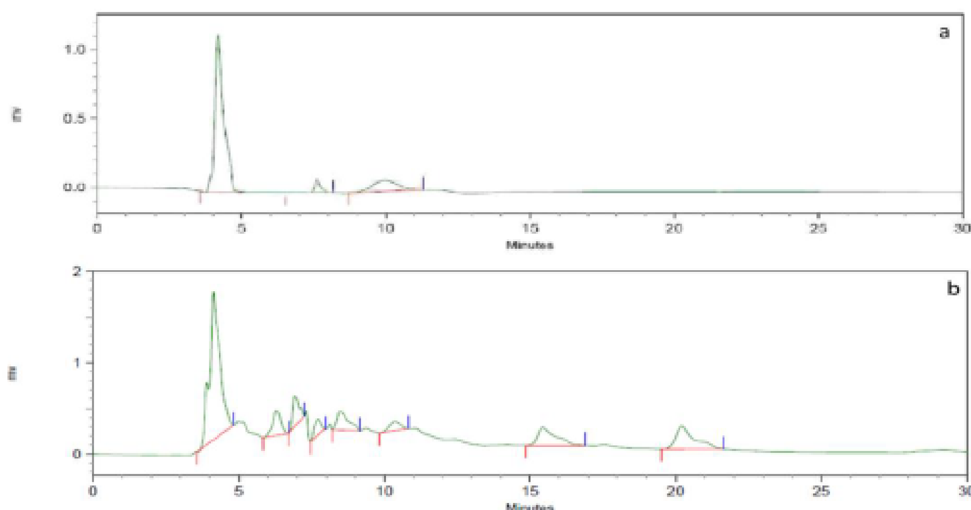

Figure 7 HPLC Chromatogram of a) standard solution of lactic acid b) culture supernatant of *P. pentosaceus* LB-CC

The antimicrobials secreted by lactic acid bacteria are mainly organic acids produced from the fermentation of sugars, which leads to the typical low pH of fermented foods. This low pH is able to inhibit the growth of most pathogens (**Boskey *et al*., 2001**). **Ribeiro *et al*. (2014)** estimated lactic acid concentration produced by *Pediococcus acidilactici* B14 which was found to be 1.1% and responsible for the antimicrobial potential of the strain. **Vodnar *et al*. (2010)** evaluated lactic acid production by probiotic bacteria on model MRS medium during fermentation processes using HPLC and found that probiotic bacteria viz. *Lactobacillus plantarum* achieved lactic acid concentration close to 6.08 g L^{-1}, *Lactobacillus casei* 6.16 g L^{-1}, *Bifidobacterium infantis* 7.09 g L^{-1} and *Bifidobacterium breve* 6.17 g L^{-1} after 78 h of fermentation resulting to their antimicrobial potential.

Bacteriocin production

The bacteriocin activity of the probiotic isolate was evaluated by assaying serial two-fold dilutions of acid neutralized and catalase treated culture filtrate supernatant (CFS) against *L. monocytogenes* and maximum bacteriocin production was observed at 18 h of growth cycle with 666 AU/ml activity units rendering its potential to be used as a safe and efficient bio-preservative as compared to harmful chemical preservative in food product. As the activity was lost after treatment with trypsin, this suggests that the activity was caused by bacteriocin produced by the isolates.

H_2O_2 production

Probiotic isolates *P. pentosaceus* LB-CC was screened for H_2O_2 production and has been reported to produce 0.52 g L^{-1}. Hydrogen peroxide is normally produced by vaginal lactobacilli isolates, but may also be associated with intestinal lactobacilli or those living in the environment (**Pascual *et al*., 2006; Martin and Suarez, 2010**). This trait is rarely reported in probiotic LAB isolated from fermented food products. In this study, *P. pentosaceus* LB-CC has been reported for the first time to produce H_2O_2 as an antimicrobial agent against food spoilage pathogens. Thus, this trait may be beneficial in improving vaginal health and in preventing urinogenital infections.
On the basis of these results, it can be hypothesized that the antagonistic activity of this strain relies on acidity, lactic acid, H_2O_2 and other antimicrobial compounds (bacteriocins), thus revealing its potential and safe use as biopreservative agents in food and fermentation industry. Efficacy and effectiveness of antimicrobial potential of *Pediococcus* spp. have been studied widely. **Mandal *et al*. (2008)** studied the antagonistic potential of *P. acidilactici* LAB5 against some food spoilage and human pathogenic bacteria and proved the antagonistic efficacy of antibacterial substance secreted by the strain i.e. bacteriocin against pathogenic bacteria viz. *Listeria, Staphylococcus, Streptococcus, Enterococcus* and *Leuconostoc*. *P. pentosaceus* 2A2 and 1A6 isolated from Indonesian local beef exhibited antibacterial activity against *E. coli* ATCC25922, EPEC, *S. typhimurium* ATCC14028 and *S. aureus* ATCC25923 (inhibition zones 5.5-14 mm) (**Arief *et al*., 2015**).

CONCLUSION

Pediococcus pantosaceus LB-CC isolated from Lasoda bari has been evaluated for its probiotic potential and was found resistance to low pH and bile salts (0.3%) and simulated gastric and intestinal conditions, was able to produce bacteriocin and lactic acid against a number of serious food borne and spoilage causing microorganisms. The susceptibility to selected eleven antibiotics, inability to produce gelatinase and DNase and non-hemolytic nature revealed its safe status for further use in food and fermentation industry. However, further evaluation of its beneficial health effects on human beings will boost the application of the strains in food and pharmaceutical industry.

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