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

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Full Length Research Paper

Lactic acid bacteria associated with the digestive tract and skin of Sea bream (*Sparus aurata*) cultured in Tunisia

Ouissal Chahad Bourouni[1], Jorge Barros-Velàzquez[2], Pilar Calo-Mata[2] and Monia El Bour[1]*

[1]Institut National des Sciences et Technologies de la Mer (INSTM), Rue 2 Mars 1934, 2025 Salammbô; Tunis, Tunisia.
[2]Department of Analytical Chemistry, Nutrition and Food Science, LHICA, School of Veterinary Sciences, University of Santiago de Compostela, E-27002 Lugo, Spain.

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Thirty-seven (37) enterococcal isolates were recovered from the skin and intestines of the sea bream (*Sparus aurata*), the most economically important fish species of the Mediterranean sea from Tunisian fish farming sites, to investigate their antimicrobial potential. All isolates were identified to the species level using genotypic tools. An investigation employing 16S rDNA sequencing in combination with randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) highlighted the predominance of the *Enterococcus faecium* (46%) and *E. faecalis* (19%) species. Other species, such as *E. sanguinicola* (3 strains), *E. casseliflavus* (3 strains), *E. gallinarum* (2 strains), *Carnobacterium* sp. (1 strain), *Aerococcus viridans* (2 strains) and *Vagococcus carniphilus* (2 strains) were also identified. The susceptibility to different antibiotics in addition to the antibacterial activities were investigated for all species identified. The isolates were sensitive to vancomycin but were resistant to several antibiotics relevant for therapy in human and animal medicine. Antibacterial profiles assayed against 39 bacterial indicators (including food-borne and fish pathogenic bacteria in aquaculture as well as other spoilage bacteria) showed that 46% of the isolates exhibited a large inhibition spectrum mainly towards *Listeria monocytogenes*, *Staphylococcus aureus*, *Aeromonas hydrophila, Aeromonas salmonicida, Vibrio anguillarum* and *Carnobacterium* strains. Therefore, highly inhibitory enterococcal strains could potentially be used as probiotics in sea bream and other farming fish fields.

Key words: Enterococcus, lactic acid bacteria, aquaculture, probiotic, sea bream.

INTRODUCTION

The frequent usage of antimicrobial agents has led to the development of multiple antibiotic resistance (MAR) in bacteria and has reduced the efficacy of antibiotic treatment for human and animal diseases (Tendencia and de la Pena, 2001; Pandiyan et al., 2013). Several studies implicated the use of antimicrobials in the fish farming sector and its environment for the prevention and treatment of animal and plant infections as well as for

*Corresponding author. E-mail: monia.elbour@instm.rnrt.tn. Tel: 00216. Fax: 00216 71732622; 00216 71277735.

promoting growth (Serrano, 2005; Kümmerer, 2009; Martinez, 2009).

The culture practices for most farmed fish species are mostly semi-intensive or intensive and farms are often affected by widespread antibiotic resistance in pathogens (*Aeromonas hydrophila, Aeromonas salmonicida, Edwardsiella tarda, Edwardsiella icttaluri, Vibrio anguillarum, Vibrio salmonicida, Pasteurella piscida* and *Yersinia ruckeri*), which are currently treated with antibiotics (Ben Kahla-Nakbi et al., 2009). Thus, replacing drugs with effective and inexpensive probiotics is necessary to avoid resistance in fish farming sites and antibiotic residues in fish flesh destined for human consumption (Rengpipat et al., 2008).

Lactic acid bacteria (LAB) belong to the bacterial communities present in the normal intestinal flora of fish and exhibit probiotic properties for aquaculture applications. Previous studies on several fish farming applications have shown the antagonistic properties of LAB on fish pathogens (Gatesoupe 1991; Ringo et al., 1995; Gonzalez et al., 2000; Vijayabaskar and Somasundaran, 2008; Rengpipat et al., 2008).

Within the LAB group, *Enterococcus spp*. are widespread in the gastrointestinal tract of mammals, birds, reptiles, insects and are found in the intestinal contents of several healthy fish species and therefore they could be amended to animal food as probiotics to contribute to the health of farmed fish (Campos et al., 2006; Calo-Mata et al., 2007).

Gilt-head sea bream (*Sparus aurata*), which together with sea bass (*Dicentrarchus labrax*) represent the main fish species with high economic value cultured in Mediterranean aquaculture and the main marine fish farmed in Tunisia, is affected by infectious diseases and the abusive use of antibiotics (Zorrilla et al., 2003; Ben Kahla-Nakbi et al., 2007). Even though it is well known that intestinal microflora, especially LAB, might influence the growth and health of farmed fish, there is no information available to date about the composition of the intestinal microflora in the sea bream that are widely cultured in Tunisia. Thus, the present study was firstly designed to investigate the presence and type of LAB of both the skin and gastrointestinal tract of farmed sea bream and to inquire about their bioactive potential against bacterial pathogens. To do so, we have characterised a large collection of sea bream LAB by phenotypic and genotypic analysis (including 16S rRNA sequencing and RAPD-PCR) and carried out the screening of their antimicrobial susceptibility patterns and their ability to produce antibacterial compounds against spoilage and fish pathogenic bacteria.

MATERIALS AND METHODS

Fish and experimental conditions

Gilt-head sea bream (*S. aurata*) specimens were collected from a fish farm in Hergla (central coast of Tunisia). Fish specimens were sampled in a water-ice mixture and kept in ice for 3 h until they arrived at our laboratory. A total of 30 fish specimens with body weights of 180-220 g were examined. Skin patches (2x1 cm^2) were aseptically excised and the intestinal content was removed by dissecting the fish, removing the intestines (to the pyloric caeca) and squeezing out the contents. The gut contents appeared as faecal matter. All samples were weighed and homogenised for 1 min in sterile plastic bags and a Stomacher (Seward, London, United Kingdom). Homogenates of skin or gut were serially diluted in 0.9% saline solution, and 0.1 ml volumes of appropriate dilutions were spread on the surface of MRS (de Man, Rogosa and Sharpe medium) and M17 plates (Oxoid, Ltd., London, UK). The plates were incubated aerobically for 48-72 h at 30°C, and the isolated colonies with typical characteristics, namely pure white and small (2-3 mm in diameter) with entire margins, were picked from each plate and transferred to MRS broth or M17 broth (Oxoid) for experimental use.

Phenotypic characterisation of the bacterial strains

Pure cultures of all the isolates were subjected to the standard tests: colony morphology, cell morphology, motility, Gram stain and the production of cytochrome oxidase and catalase, fermentation tests of glucose and lactose, H_2S and gas production, the ability to grow at 10°C and 45°C in media (Brain Heart Infusion) containing 6.5% NaCl at pH 9.6 (Schleifer and Kilpper-Bälz, 1984). All the Gram-positive bacteria belonging to the LAB group were further tested by means of miniaturised API 50 CH biochemical tests (BioMérieux, Marcy L'Etoile, France). The results of the identification tests were interpreted using the APILAB PLUS software, version 4.0 (BioMérieux).

Genetic identification of LAB strains

DNA from the LAB was isolated from the pellets formed after spinning 1 ml of overnight cultures in MRS broth at 7500 rpm for 10 min. Each pellet was re-suspended in 180 µl of lysis buffer (20 mM Tris-Cl pH8, 2 mM EDTA, 1.2% triton X-100, 20 mg/ml lysozyme). Each 10 ml of lysis buffer was prepared by mixing 4 ml of lysozyme (10 mg/ml, in bi-distilled water), 4 ml of 50 mM Tris-HCl, 200 µl of 100 mM EDTA, 120 µl of Triton X-100 and 1.68 ml of Milli-Q water. All the reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). After an incubation step at 37°C for 2 h, 25 µl of proteinase K (10 mg/ml) (Sigma) was added, followed by incubation at 70°C for 30 min. Then, the bacterial DNA was purified from each extract by means of a DNeasy tissue minikit (QIAGEN Inc., Valencia, CA, USA), based on the use of micro-columns. The concentration of purified DNA extract was determined by measuring the fluorescence that developed by using a Quanti-iT kit and a Qubit fluorimeter (Invitrogen).

The genetic characterisation of LAB isolates was performed by a PCR amplification of 16S rDNA using the universal set of primers p8FPL (forward: 5'-AGTTTGATCCTGGCTCAG-3') and p806R (reverse: 5'-GGACTACCAGGGTATCTAAT-3') that yield an 800 bp PCR product (McCabe et al., 1995). The amplification conditions were as follows: a previous denaturing step at 94°C for 7 min was coupled to 30 cycles of denaturation (94°C for 1 min), annealing (55°C for 1 min) and extension (72°C for 1 min) and to a final extension step at 72°C for 15 min. All the amplification assays comprised 100 ng of the template DNA, 25 µl of a master mix (BioMix, Bioline Ltd., London, UK), including the reaction buffer, dNTPs, magnesium chloride and *Taq* DNA polymerase, PCR water (Genaxis, Montigny le Bretonneaux, France), and 5 µl of each oligonucleotide primer to achieve a final volume of 50 µl. All PCR assays were carried out on a MyCycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA). The PCR products were visualized in 2.5% horizontal agarose (MS-8, Pronadisa, Madrid,

Spain) gels.

Prior to sequencing, the PCR products were purified by means of an ExoSAP-IT kit (GE Healthcare, Uppsala, Sweden). Direct sequencing was performed with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The same primers used for PCR were employed for the sequencing of both strands of the PCR products. The sequencing reactions were analysed in an automatic sequencing system (ABI 3730 XL DNA Analyser, Applied Biosystems) with the POP-7 system. Sequence homologies were searched using the BLAST tool (National Centre for Biotechnology Information). The alignment of the new sequences with other ones present in GenBank was accomplished using the ClustalX software (Larkin et al., 2007). Phylogenetic and molecular evolutionary analyses were conducted with the MEGA software (Kumar et al., 2008), using the neighbour-joining method (Saitou and Nei, 1987) and the Kimura 2-parameter with 1000 bootstrap replicates to construct distance-based trees.

RAPD-PCR reaction

RAPD-PCR was performed using 200 ng of the template DNA and 25 µl of a master mix (BioMix, Bioline Ltd., London, UK), including the reaction buffer, dNTPs, magnesium chloride and Taq DNA polymerase and PCR water (Genaxis, Montigny le Bretonneaux, France) and 14 pmol of M13 (5´-GAGGGTGGCGGTTCT-3´) (Andrighetto et al., 2001) to achieve a final volume of 50 µl. The amplification reactions were performed using a thermal cycler from Applied Biosystems (GeneAmp-PCR System 2700). The following reaction conditions were used: initial denaturalisation at 94ºC for 5 min, followed by 33 cycles at 94ºC for 60 s, annealing at 45ºC for 60 s, extension at 72ºC for 60 s, and final extension at 72ºC for 15 min. Ten µl of the PCR products were separated and visualised using 1.5% horizontal agarose gels (MS-8, Pronadisa, Madrid, Spain) in a solution of 1XTAE buffer (Tris-acetate-EDTA) and ethidium bromide (10 mg/ml) with electrophoresis at 80 V. The 123-bp DNA ladder (DNA LADDERS D 5042, Sigma) was used as a size marker. To check the reproducibility, the PCR assays were performed at least three times each. In each reaction, a tube without the template DNA was included as a negative control.

Microbial sensitivity towards antibiotics

The bacterial sensitivity was determined by the agar diffusion method according to Chabbert (1982) using the following 16 antibiotics selected as representatives of the different classes of antimicrobial agents relevant for therapy in human and animal medicine: vancomycin (30µg), penicillin G (10 UI), amoxicillin (25 µg), oxacilin (5 µg), cefoxitin (30 µg), ceftriaxone (30 µg), streptomycin (10 UI), tobramycin (10 µg), neomycin (30 UI), chloramphenicol (30 µg), tetracycline (30 UI), oleandomycin (15 UI), nitrofurantoin (300 UI), trimethoprim-sulphamide (25 µg), rifampicin (30 µg) and oxolinic acid (30 µg). Five ml of overnight culture in MRS broth, of the LAB strains was spread out on the surface of Mueller-Hinton agar plates (Oxoid). Then, the paper disks that were impregnated with the antimicrobial agents were placed onto the agar plate. After overnight incubation at 20°C, the diameter of the zone of inhibition of bacterial growth around each disk was measured. Based on the zones of inhibition, a qualitative report of “susceptible”, “intermediate” or “resistant” was determined for the tested bacteria according to the French National Guidelines (Comité de l’Antibiogramme de la Société Française de Microbiologie, 1996).

Antibacterial activity of LAB strains

The potential bacteriocin-producing strains were screened against a range of 39 indicator pathogenic and spoilage microorganisms (Table 1). The detection of bacteriocin activity in LAB strains was initially screened by means of a standardised agar disk diffusion method. Briefly, Muller-Hinton (Oxoid) agar plates were seeded with a bacterial lawn of each indicator strain at a 10^5 CFU/ml concentration. Then, extracellular extracts were prepared by centrifugation, at 7,000 rpm for 15 min, of 48-h culture in MRS of each strain and the cell-free extract was sterilized by filtration through 0.22 µm (Millex GS, Millipore, St. Quentin, France). Twenty µl of each LAB strain extracellular extract were placed on 6-mm sterile disks (Oxoid) that had previously been placed on the agar plates. The plates were incubated overnight at 37°C, and the antimicrobial activity was detected by the appearance of translucent halos in the bacterial lawn surrounding the disks. A nisin-producing *L. lactis* strain was included as a positive control for the antimicrobial activity.

RESULTS

Isolation of microorganisms

From the different samples of sea bream, 37 microbial isolates were examined; 11 of these strains were isolated from the skin and 26 strains were from the intestinal content. The physiological and biochemical characteristics of the isolated LAB strains are shown in Table 2. Thus, all the isolates were Gram-positive, catalase-negative, non-motile, non-spore-forming and chain-forming cocci, able to ferment glucose and to grow at 10ºC and 45ºC and in media containing 6.5% NaCl. All the strains produced acid from glucose, fructose, arbutin, esculin, maltose and trehalose, but not from arabinose, inositol, starch, rhamnose, dulcitol, inuline, xylitol, turanose, lyscose, fucose, arabitol, ceto-gluconate, erythritol, xylose or adonitol.

Identification

The genomic DNA of all the isolates was purified and ca. 800-bp fragments of their 16S rDNA were amplified and sequenced. The alignment of the 16S rRNA sequences showed that all the strains exhibited very high homology (≥ 95%) among themselves and with other *Enterococcus* strains deposited in the GenBank database. The results of the alignements allowed the classification of nine intestinal strains as *E. faecium*, seven strains as *E. faecalis*, three strains as *E. casseliflavus*, one strain as *Enterococcus gallinarum*, two strains as *E. sanguinicola,* one strain as *Carnobacterium* sp., two strains as *Aerococcus viridans* and two other strains as *Vagococcus carniphilus*. However, ten enterococci could not be identified to the species level but could only be identified to the genus level (Table 2). From the skin, only four different species were isolated (*E. faecium, E. faecalis, A. viridans* and *Carnobacterium* sp.).

The dendrogram derived from the sequence homology comparison of 16S rRNA gene sequences of isolates with respect to the reference sequences from GenBank is

Table 1. Pathogenic and spoilage indicator microorganisms used to test the antibacterial activities of LAB isolates.

Code	Species	Origin
AmH01	*Aeromonas hydrophila*	ATCC 7966
BaC23	*Bacillus cereus*	ATCC 14893
BaP31	*Bacillus pumilus*	ATCC 7061
BaS05	*Bacillus Subtilis ssp. Spizizenii*	ATCC 6633
BxT01	*Brochotrix thermosphacta*	ATCC 11509
CbD21	*Carnobacterium divergens*	ATCC 35677
CbM01	*Carnobacterium maltaromaticum*	LHICA collection
EbA01	*Enterobacter aerogenes*	ATCC 13048
EbC11	*Enterobacter cloacae*	ATCC 13047
HaA02	*Hafnia alvei*	ATCC 9760
KlOx11	*Klebsiella oxytoca*	ATCC 13182
KlP02	*Klebsiella planticola*	ATCC 33531
KlPn21	*Klebsiella Pneumoniae* ssp. *pneumoniae*	ATCC 10031
Lb30A	*Lactobacillus saerimneri*	LHICA collection
MoM02	*Morganella morganii* ssp. *morganii*	ATCC 8076H
PhD11	*Photobacterium damselae*	ATCC 33539
PrM01	*Proteus mirabilis*	ATCC 14153
PrP11	*Proteus penneri*	ATCC 33519
PrV21	*Proteus vulgaris*	ATCC 9484
PsF12	*Pseudomonas fluorescens*	ATCC 13525
PsFr51	*Pseudomonas fragi*	ATCC 4973
PsG21	*Pseudomonas gessardii*	LHICA collection
SrM53	*Serratia marcescens* ssp. *marcescens*	ATCC 274
SyE21	*Staphylococcus xylosus*	ATCC 35983
SyX11	*Stenotrophomonas maltophilia*	ATCC 29971
StM03	*Staphylococcus aureus*	ATCC 13637
59	*Staphylococcus aureus*	ATCC 9144
4521	*Lysteria monocytogenes*	ATCC 35845
4032	*Lysteria monocytogenes*	NCTC 11994
1112	*Lysteria monocytogenes* 1112	LHICA collection
CI34.1	*Pseudomonas anguilliseptica*	Seabream*
ACR5.1(AS)	*Aeromonas salmonicida*	Turbot*
CI52.1(VCI)	*Vibrio anguillarum*	Seabream*
ACC30.1	*Photobacterium damselae* ssp. *piscida*	Sole*
V62	*Vibrio anguillarum*	Seabream**
VF	*Vibrio anguillarum*	Seabass***
AF	*Aeromonas salmonicida*	Seabass***
V90.11.287(V287)	*Vibrio anguillarum*	Seabass****
AH2	*Pseudomonas fluorescens*	*Lates niloticus*****

*Strains provided by Pr. J. L. Romalde (Spain). ** Strain provided by Pr. G. Breuil (France). ***Strains provided by Pr. J. C. Raymond (France). ****Strains provided by Pr. L. Gram (Denmark).

shown in Figure 1. The dendrogram suggests a close relationship between our isolates and the reference strains. According to this classification, the ten isolates that were identified to the genus level could be assigned to *E. faecium* (8 strains), *E. gallinarum* (1 strain) and *E. sanguinicola* (1 strain).

RAPD-PCR analysis

Further genetic intra-specific characterisation of the isolates was performed by RAPD-PCR analysis with M13 primers. The dendrogram derived from the combination of amplification profiles obtained with primers M13 is

Table 2. Biochemical and phenotypical tests of the LAB isolates

Stain	Organe	Oxydase	Gaz	H2S	Glycerol	L-Arabinose	Ribose	D-Xylose	Galactose	Manose	Sorbose	Mannitol	Sorbitol	a-Methyl-D-Mannosie	a-Methyl-D-Glucoside	N-Acetyl-Glucosamine	Amygdaline	Salicine	Cellobiose	Lactose	Melibiose	Saccharose	Melezitose	Raffinose	Glycogene	Gentiobiose	D-Tagatose	L-Fucose	L-Arabitol	Gluconate	Identification
UPAA5	Skin	-	-	-	-	+	+	-	+	+	-	+	+	-	-	+	+	+	+	+	+	-	-	-	-	+	+	-	-	+	*E. faecium*
UPAA9	Intestine	-	+	+	-	+	+	+	+	+	-	+	+	-	-	+	+	+	+	+	+	+	-	+	-	+	+	-	-	+	*E. cassel.*
UPAA11	Intestine	-	-	-	-	-	+	+	+	+	-	+	-	-	+	+	+	+	+	+	+	+	-	+	-	+	-	-	-	-	*E. faecium*
UPAA13	Intestine	-	-	-	+	-	+	-	+	+	-	+	-	+	-	+	+	+	+	+	-	+	-	-	-	+	-	-	+	-	*E. faecalis*
UPAA21	Intestine	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*E. faecalis*
UPAA22	Skin	+	+	-	-	+	+	-	+	+	+	+	+	-	-	+	+	+	+	+	+	-	-	-	-	+	+	-	-	+	*E. faecium*
UPAA26	Intestine	-	-	-	-	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+	+	-	-	+	+	-	-	+	*E. faecalis*
UPAA34	Skin	-	-	-	-	+	+	-	+	+	-	+	+	-	-	+	+	+	+	+	+	-	-	-	-	+	+	-	-	+	*E. faecium*
UPAA35	Intestine	-	-	-	-	+	+	-	+	+	-	+	+	-	-	+	+	+	+	+	+	-	-	-	-	+	+	-	-	+	*E. faecium*
UPAA38	Skin	-	-	-	+	-	+	-	+	+	-	+	+	-	-	+	+	+	+	+	-	+	+	-	-	+	+	-	-	+	*E. faecalis*
UPAA39	Intestine	-	-	-	-	+	+	+	+	+	-	-	-	-	-	+	-	+	+	+	+	+	-	-	-	+	+	-	-	-	*E. faecium*
UPAA40	Intestine	-	-	-	-	+	+	-	+	+	-	+	+	-	-	+	+	+	+	+	+	-	-	-	-	+	+	-	-	+	*E. faecium*
UPAA44	Skin	-	-	-	-	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+	-	+	-	+	+	-	-	-	*E. faecium*
UPAA45	Intestine	-	-	-	-	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+	-	+	-	+	+	-	-	-	*E. faecium*
UPAA46	Intestine	+	-	-	+	-	+	+	-	+	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	*V. carniphilus*
UPAA49	Intestine	-	+	-	+	+	+	+	+	+	-	+	-	-	-	+	+	+	+	+	+	+	-	+	-	+	-	-	-	-	*E. sang.*
UPAA51	Intestine	+	-	-	-	+	+	+	+	+	-	+	-	-	-	+	+	+	+	+	-	+	-	+	-	+	-	-	-	+	*V. carniphilus*
UPAA52	Skin	-	-	-	+	-	+	-	+	+	-	+	+	-	-	+	+	+	+	-	-	+	+	-	-	+	+	-	-	+	*E. faecalis*
UPAA60	Intestine	-	-	-	+	-	+	-	+	+	-	+	-	+	-	+	+	+	+	+	-	+	-	-	-	+	-	-	-	-	*E. faecium*
UPAA61	Skin	-	+	-	-	-	+	-	+	+	+	+	-	+	-	+	+	+	+	+	+	+	-	+	-	+	-	-	-	-	*E. faecium*
UPAA66	Skin	-	-	-	-	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+	-	+	+	+	+	-	-	-	*E. faecium*
UPAA68	Intestine	-	-	-	+	-	+	-	-	+	-	+	-	-	-	+	+	+	+	-	-	-	-	-	-	+	-	+	-	-	*A. viridans*
UPAA71	Intestine	+	-	-	+	-	+	-	-	+	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	+	-	+	-	-	*E. sang.*
UPAA72	Intestine	-	-	-	+	+	+	-	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	*E. sang.*
UPAA75	Intestine	-	-	-	+	-	+	-	+	+	-	+	+	-	-	+	+	+	+	+	-	+	+	-	-	+	+	-	-	+	*E. faecalis*
UPAA77	Skin	-	+	+	-	-	+	-	+	+	-	+	-	+	-	+	+	+	+	+	+	+	-	+	-	+	-	-	-	-	*Carnob. sp.*
UPAA82	Intestine	-	-	-	-	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	*E. gallinarum*
UPAA85	Intestine	-	-	-	-	-	+	-	+	+	-	+	-	+	-	+	+	+	+	+	+	+	-	+	-	+	-	-	-	-	*E. faecium*
UPAA86	Skin	-	-	-	+	-	+	-	+	+	-	+	+	+	-	+	+	+	+	+	-	+	+	-	-	+	+	-	-	-	*A. viridans*
UPAA87	Intestine	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	*E. faecium*
UPAA89	Intestine	+	-	-	+	-	+	-	+	-	-	+	-	-	-	+	+	+	+	+	-	+	-	-	-	+	-	-	-	+	*E. faecium*
UPAA100	Intestine	-	-	-	+	-	+	-	+	+	-	+	-	-	-	+	+	+	+	+	-	+	-	-	-	+	-	-	-	+	*E. faecalis*
UPAA102	Skin	-	+	+	-	-	+	-	+	+	-	+	-	+	-	+	+	+	+	+	+	+	-	+	-	+	-	-	-	-	*E. faecalis*
UPAA103	Intestine	-	-	-	-	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	-	-	+	+	-	-	-	*E. cassel.*
UPAA104	Intestine	-	-	-	-	+	+	+	+	+	-	+	+	-	-	+	+	+	+	+	+	+	-	+	-	+	+	-	-	+	*E. cassel.*
UPAA106	Intestine	-	-	-	-	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	*E. gallin.*
UPAA110	Intestine	+	-	+	+	-	+	+	-	+	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	*E. faecium*

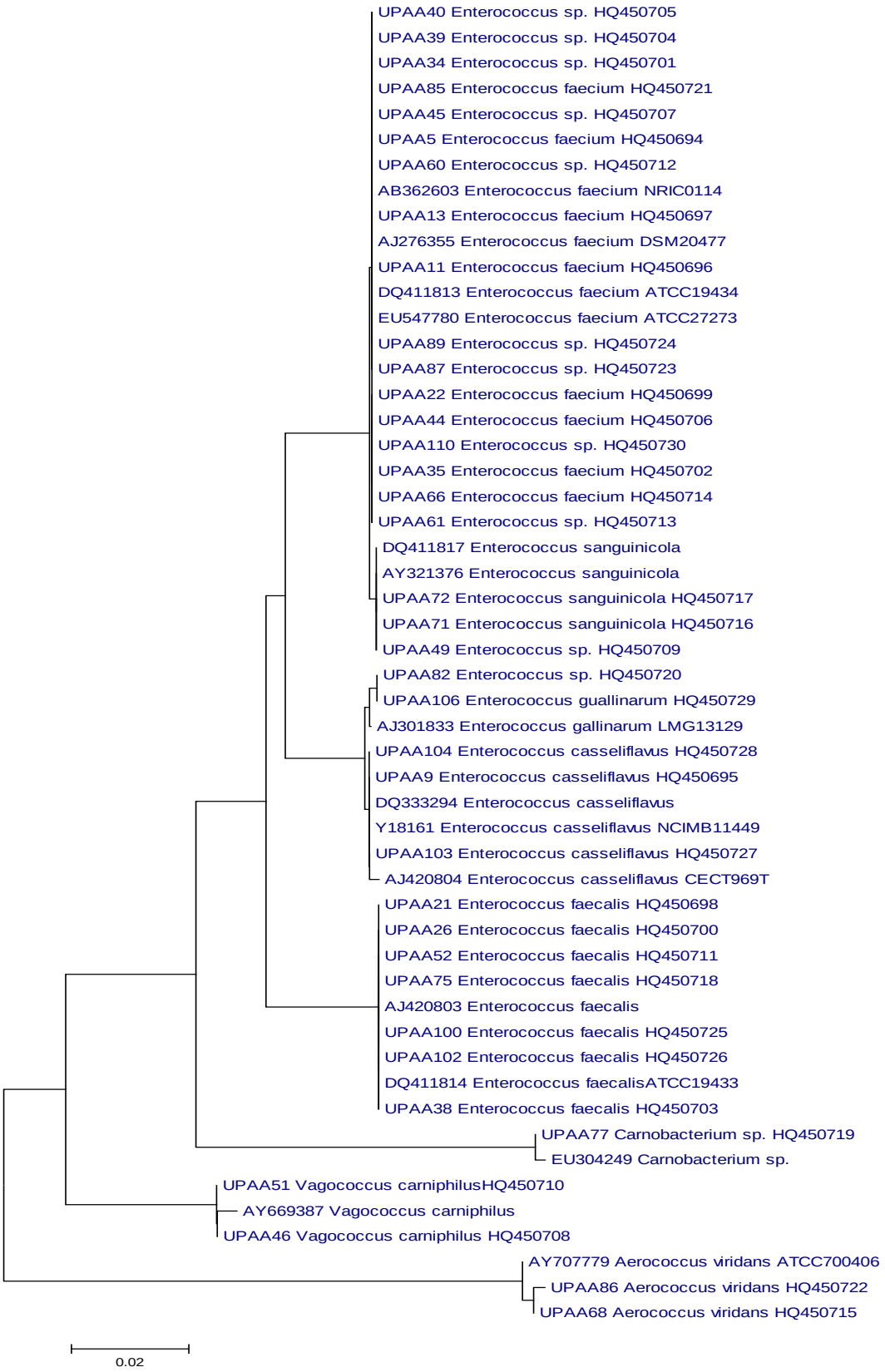

Figure 1. Phylogenetic relationships according to the partial sequencing of the 16S rDNA gene of LAB isolates and reference strains from GenBank by means of the neighbor-joining method. (X) GenBank accession numbers of the LAB isolates.

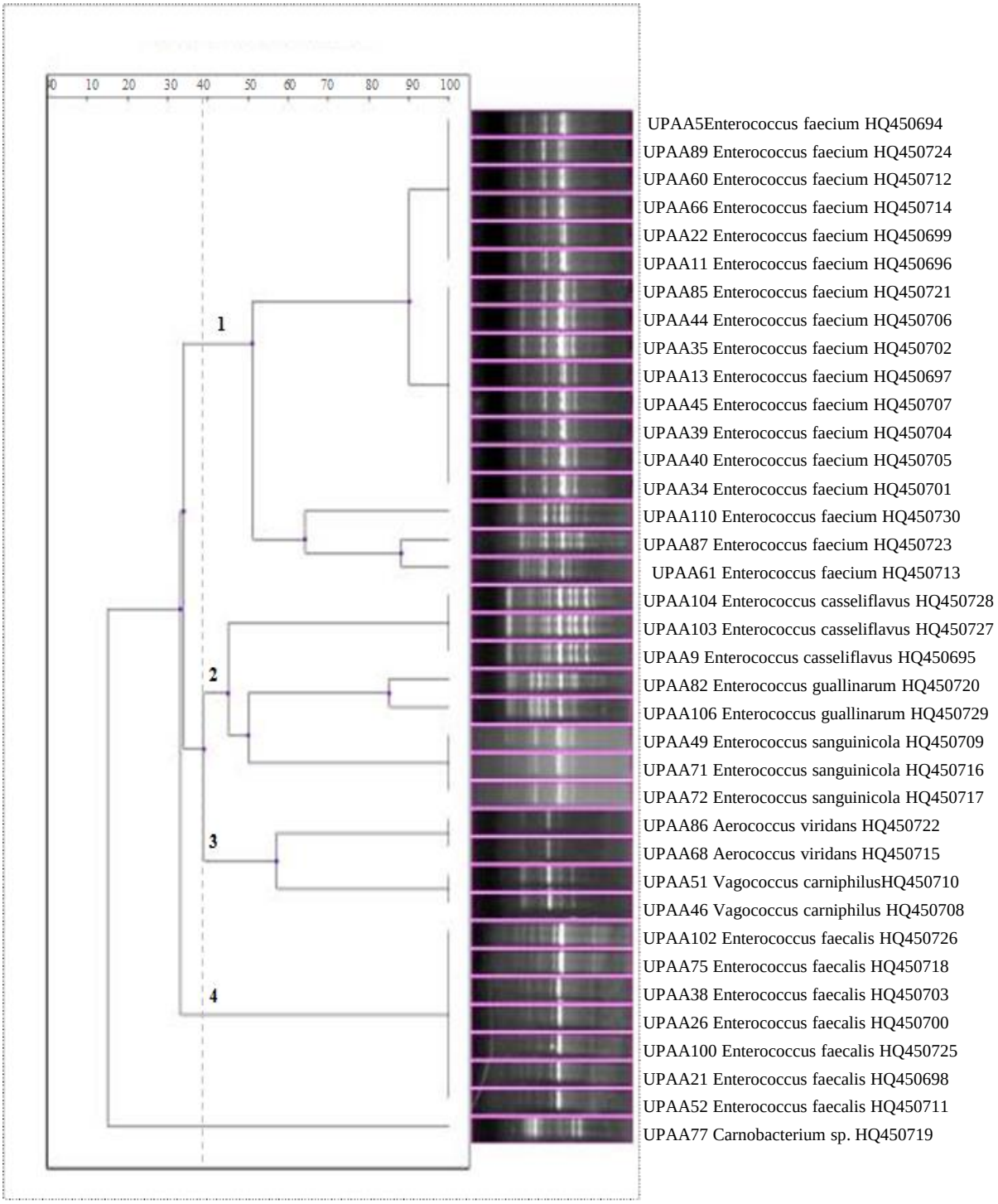

Figure 2. RAPD-PCR patterns of the isolates obtained by using the primers M13, and dendrogram obtained by UPGMA of correlation value of merged normalised RAPD-PCR patterns.

shown as Figure 2. Thus, the RAPD analysis with M13 primers yielded a clear discrimination of the different *Enterococcus* species isolated, allowing their grouping into clusters corresponding to each species. At a similarity level of 40%, arbitrarily chosen for the defining species, four main clusters were observed. The Cluster 1 grouped isolates belonged to the species *E. faecium*. Cluster 2 could be divided into three subclusters, each of them grouping isolates belonging to *E. casseliflavus, E. gallinarum* and *E. sanguinicola*, respectively. The third

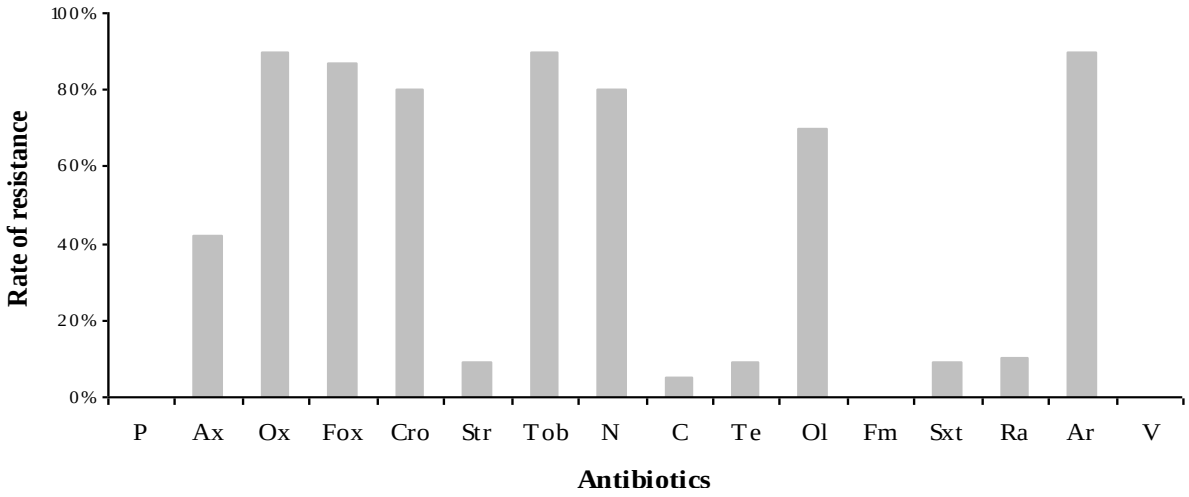

Figure 3. Profiles of resistance obtained for the farmed sea bream LAB strains tested against the 16 antimicrobial agents. P, penicillin; Ax, amoxicillin; Ox, oxacillin; Fox, cefoxitin; Cro, ceftriaxon; Str, streptomycin; Tob, tobramycin; N, neomycin; C, chloramphenicol; Te, tetracycline; Ol, oleandomycin; Fm, furans; Sxt, trimethoprim-sulphamide; Ra, rifampicin; Ar, oxolinic acid; V, vancomycin.

cluster also contained two subclusters that grouped isolates belonging to *A. viridans* and *V. carniphilus.* Finally, a fourth cluster grouped isolates belonging to *E. faecalis* species. The strain *Carnobacterium sp*. clustered as an independent strain. It is particularly interesting to note that the grouping of the isolates with the RAPD analysis was in agreement with the classification provided by 16S RNA sequencing.

After the comparison of the amplification profiles obtained for the isolates that were identified to the genus level with those generated for others strains, it was possible to assign 8 isolates to *E. faecium*, one isolate to *E. gallinarum* and one to *E. sanguinicola,* which confirmed the data provided by the dendrogram generated by 16S RNA sequencing. These results confirmed the data resulting from the dendrogram generated by the 16S RNA phylogenetic analysis.

Microbial sensibility towards antibiotics

All the strains tested were resistant to at least three of the antibiotics. Thus, resistance to oxacillin, cephalosporins (cefoxitin, ceftriaxon), aminoglucosids (tobramycin and neomycin), macrolids (oleandomycin) and oxolinic acid were common among the isolates (Figure 3). In contrast, penicillin, streptomycin, phenicol, tetracyclin, rifampicin, trimethoprim-sulphamid and nitrofurantoin were the most active antibiotics against the majority of the LAB isolates. Nevertheless, it is well known by now that the administration of nitrofurantoin is banned in fish and shellfish farming. Interestingly, all the strains were sensitive to vancomycin.

The resistance patterns of the enterococcal isolates indicated a considerable diversity of strain-specific antibiotypes. Thus, up to 18 different antibiotypes were characterised, including those with resistance to three to ten antimicrobial agents (Table 3). Five different resistance types against seven antibiotics, four resistance types against eight antibiotics, three resistance types against six and nine antibiotics and two resistance types against ten antibiotics were characterised (Table 3).

An analysis of the phenotypic relationships among the enterococci isolated from the skin and intestinal content of the fish was also carried out and showed that, among the 28 antimicrobial resistance patterns obtained, nine were specific to the isolates recovered from the intestines of the fish and that five patterns were specific of those recovered from the fish skin.

Antibacterial activity by LAB isolates

All the isolates were assayed for inhibitory production against 39 Gram-positive and Gram-negative indicator bacteria, including food-borne and fish pathogenic bacteria and other spoilage bacteria (Table1). Seventeen strains (46%) exhibited inhibitory activity against a large number of the indicator strains investigated (Figure 4). Greater inhibition was observed against *L. monocytogenes*, *S. aureus*, *A. hydrophila, A. salmonicida, V. anguillarum* and *Carnobacterium* strains (Table 4). The diameters of the inhibition halos were within the 6.5–20 mm range. Thus, we selected 12 strains that strongly inhibited a large number of indicators and generated inhibitory zones with diameters larger than 11 mm for future studies and to evaluate their potential use as probiotics.

Table 3. Antibiotypes of the LAB strains isolated from farmed sea bream.

Strain	Lab codes	No. of resistance	Type of antimicrobial agents
Enterococcus sp.	UPAA 60	10	N-RA-FOX-TE-OX-CRO-AX-OL-AR-TOB
Enterococcus faecuim	UPAA 66		N-SXT-FOX-OX-CRO-AX-OL-AR-STR-TOB
Enterococcus faecium (6 strains)	UPAA 11/13/22/85/45/61	9	N-FOX-OX-CRO-AX-OL-AR-STR-TOB
Enterococcus faecalis	UPAA 21		N-FOX-OX-CRO-AX-OL-AR-STR-TOB
Enterococcus sanguinicola	UPAA 72		N-FOX-OX-CRO-AX-OL-AR-STR-TOB
Enterococcus faecalis	UPAA 26		N-FOX-TE-OX-CRO-AX-AR-STR-TOB
Enterococcus gallinarum	UPAA 82		N-FOX-TE-OX-CRO-AX-AR-STR-TOB
Enterococcus faecuim (2 strains)	UPAA 89/34		N-FOX-TE-OX-CRO-AX-AR-STR-TOB
Enterococcus faecalis	UPAA 75		N-SXT-RA-FOX-OX-CRO-OL-AR-TOB
Enterococcus gallinarum	UPAA 106	8	N-FOX-OX-CRO-OL-AR-STR-TOB
Enterococcus faecalis (2 strains)	UPAA 102/38		N-FOX-OX-CRO-OL-AR-STR-TOB
Carnobacteruim sp.	UPAA 77		N-FOX-OX-CRO-OL-AR-STR-TOB
Enterococcus faecalis	UPAA 100		FOX-OX-CRO-AX-OL-AR-STR-TOB
Enterococcus faecium	UPAA 5		N-SXT-RA-FOX-OX-OL-AR-TOB
Enterococcus faecalis	UPAA 52		N-FOX-OX-CRO-AX-OL-AR-TOB
Enterococcus faecuim	UPAA 40	7	N-SXT-RA-FOX-OL-AR-TOB
Enterococcus faecuim	UPAA 39		N-OX-CRO-AX-AR-STR-TOB
Enterococcus faecuim (2 strains)	UPAA 44/35		N-FOX-OX-CRO-OL-AR-TOB
Enterococcus casseliflavus	UPAA 104		N-FOX-OX-CRO-OL-AR-TOB
Vagococcus carnophulis (2 strains)	UPAA 46/51		N-FOX-OX-CRO-AR-STR-TOB
Enterococcus faecuim	UPAA 110		N-FOX-OX-CRO-AR-STR-TOB
Enterococcus sanguinicola	UPAA 71		N-FOX-OX-CRO-AR-STR-TOB
Enterococcus sanguinicola	UPAA 49		FOX-OX-CRO-AX-AR-STR-TOB
Enterococcus casseliflavus	UPAA 103		N-C30-OX-AX-OL-AR-STR
Aerococcus viridans	UPAA 86	6	F-C30-OX-AX-OL-TOB
Enterococcus faecuim	UPAA 87		N-FOX-OL-AR-STR-TOB
Enterococcus casseliflavus	UPAA 9		N-FOX-OX-CRO-AR-TOB
Aerococcus viridans	UPAA 68	3	OL-AR-TOB

AX, amoxicillin; OX, oxacillin; FOX, cefoxitin; CRO, ceftriaxon; STR, streptomycin; TOB, tobramycin; N, neomycin; C, chloramphenicol; TE, tetracyclin; OL, oleandomycin; FM, furans; SXT, trimethoprim-sulphamide; RA, rifampicin; AR, oxolinic acid.

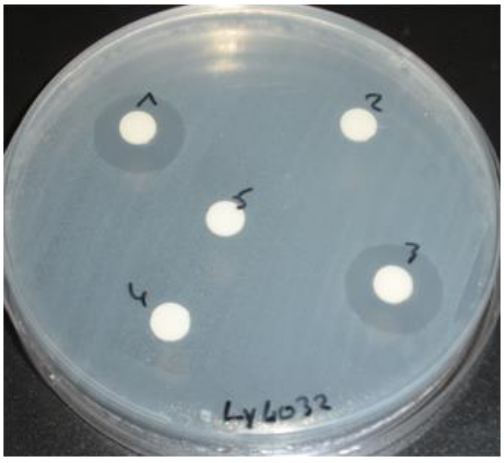

Figure 4. Antimicrobial activity of cell-free supernatants from Enterococcus isolates against *lysteria monocytogenes.* 1, UPAA34; 2, UPAA26; 3, UPAA39; 4, UPAA21 and 5, UPAA13.

DISCUSSION

The high mortality rates that occur in the larval phases of cultures of marine fish such as sea bream (*Sparus aurata*), which is one of the most valuable cultured species in Tunisia and other Mediterranean countries, cause great economic losses to aquaculture facilities in these countries. This mortality has been frequently attributed to bacterial infections (Toranzo et al., 1993; Villamil et al., 2003). Among the possible ways to prevent this problem whilst avoiding the extensive use of antibiotics, is the use of bacteria such as LAB (potential probiotics). This subject has received increasing attention during the last decade (Ringo and Gatesoupe, 1998; Gatesoupe, 1999; Vazquez et al., 2004; Anders et al., 2010). However, to our knowledge no study has described the isolation, screening and characterisation of lactic acid bacteria to be used as probiotics in sea bream or in other fish species from Tunisian aquaculture facilities. Therefore, this study was firstly designed to

Table 4. Antimicrobial activity of the enterococcal isolates against Gram-positive and Gram-negative fish pathogenic and food spoilage micro-organisms.

Producers strain	Indicators strain																								
	AmH01	BaC23	BaP31	BaS05	BxT01	CbD21	CbM01	HaA02	KlPn21	MoM02	PrV21	PsFr51	SrM53	SyX11	59	4521	4032	1112	AS	AF	V62	VCl	V321	V287	VF
UPAA5	7		8	8		10	10	6.5		7		6.5	6.5		9	9	9	8	10		9	10	8		
UPAA11	7	7				10	10		9		8						11	13	10		16	11		10	10
UPAA22	8	7				9	10		9								10	10			10	10		9	
UPAA26	10	7							9		10											10			
UPAA34	8	7	10		6.5	17	10										16	18	10	11	11	10		12	11
UPAA35	9	7	10		6.5	18	12										16	16	10	11	12	11		12	11
UPAA39	8	7	10		6.5	17	12										16	16	10	11	13	10		12	11
UPAA40	8				6.5	14	10										18	18	9	8	15	11		11	8
UPAA44						20	11										18	14	10	10	14	10			
UPAA45																	10	12	10	10	10	10			
UPAA49	9	7	8		6.5												10	10	9	10		9		10	9
UPAA61	7		9	8	6.5					7							9	8	8	9		10			
UPAA71	10	7	10		6.5												11	11	10	10	10	10		11	10
UPAA72	10	7							9		9						10	10	9	9	10	9		9	9
UPAA85	7		8											7			9	9	10	9	12	12			8
UPAA89	7		9											7			10	9	10	10	10	10			10
UPAA110	7			8	6.5		9	6.5		7							13	10	11	10	12	9			

Results are expressed as diameters of the inhibition zone in mm. Indicator strains EbA01, EbC11, KLOX11, KLP02, Lb30A, PhD11, ACC30.1, CI34.1, PrM01, PsF12, PrP11, PsG21, SyE21, StM03 were not inhibited by any LAB strain.

isolate, identify and characterise LAB associated with the skin and intestines of healthy sea bass because these LAB isolated *in situ* are normal residents and are persistent in the skin and intestines of the hosts; therefore, the host immune system should tolerate them (Tannock, 1999).

Remarkably, enterococci were found to be ubiquitous among the fish samples tested. Enterococci are part of the normal intestinal microbiota of humans and animals and are used as indicators of faecal contamination of recreational water, but they can also be isolated from natural environments that have not been contaminated by faecal material (Roberts et al., 2009). Their occurrence in fish and fish environments has been described before (Kanoe and Abe, 1988; Peterson and Dalsgaard, 2003; Michel et al., 2007). The identification of *Enterococcus* species by physiological tests has always been problematic because of their considerable phenotypic diversity (Park et al., 1999), and commercially available kits are frequently insufficient for an accurate identification (Angeletti et al., 2001). Hence, in this work phenotypic analyses were complemented with 16S rDNA phylogenetic analysis and RAPD cluster analysis. A high congruency between RAPD and phylogenetic clusters was observed in this work, which is in agreement with previous reports (Vancanneyt et al., 2002; Linaje et al., 2004). Our work identified *E. faecium* as the most commonly isolated *Enterococcus* species from European sea bream (*Sparus aurata)* (46% of

microbial isolates), followed by *E. faecalis* (19%), and, to a lesser extent *E. sanguinicola*, *E. casseliflavus* and *E. gallinarum*. Three species were also isolated: *Carnobacterium sp*. (one strain), two strains of *Aerococcus viridans* and two strains of *Vagococcus carniphilus.*

E. faecalis, E. faecium and other enterococcal species were not considered as indigenous flora of the fish gut (Ringo and Gatesoupe, 1998). However, Kanoe and Abe (1988) found high counts of *E. faecalis* and *E. faecium* in intestinal samples from marine fish, and Peterson and Dalsgaard (2003) noted the predominance of these two species among the enterococci isolated from integrated and traditional fish farms, suggesting that enterococci may be a member of the normal intestinal flora of fish. The high prevalence of *E. faecium* isolates recovered from our fish intestinal samples support this possibility. Identical results were found when we isolated LAB from a sea bass gut (*Dicentrarchus labrax*) (data not shown). Concerning the genotypic characterisation of the isolates, and as reported in previous studies (Andrighetto et al., 2001; Suzzi et al., 2000; Vancanneyt et al*.*, 2002), RAPD-PCR has been shown to be a valid and accurate method for the identification of enterococci and for detecting genetic diversity at strain level. The results obtained are in agreement with the phylogenetic analysis based on 16S rRNA sequences.

The antibiotic resistance trends among *Enterococcus* species have been extensively reviewed (Bonten et al., 2001; Franz et al., 2003). This matter has been mostly investigated for clinical and human enterococcal isolates because of their high clinical impact. In addition, a number of studies have attempted to compare the resistance spectra of different enterococci according to their human, animal or food origins (Ogier and Serror, 2008). The occurrence of antibiotic resistance among isolates seems to vary somewhat between studies and is often described to be strain- and region-dependent (Canzek et al., 2005) or may also differ according to the isolation method (Klein, 2003).

Enterococci are intrinsically resistant to low levels of penicillin, cephalosporins and aminoglycosides, and currently, these bacteria have acquired high-level resistance to vancomycin and/or aminoglycosides (Roberts et al., 2009). The *Enterococcus* spp. isolated in our study were sensitive to vancomycin, penicillin and nitrofurantoin.

Remarkably, streptomycin, phenicol, tetracyclin, rifampicin and trimethoprim-sulphamid were the most active antibiotics against the majority of the bacterial isolates that were resistant to other antimicrobials tested (oxacillin, cephalosporins, aminoglucosids, macrolids and oxolinic acid). The frequent detection of antibiotic resistance among enterococci is probably due to the increasing use of antibiotics (Bhattacherjee et al., 1988; Pathak et al., 1993; Goni-Urriza et al., 2000; Rhodes et al., 2000), which is complicated by the efficient transfer mechanisms of resistance genes via conjugative plasmids and transposons operating in this bacterial group. Therefore, antibiotic resistance, at least to vancomycin, must be evaluated in these microorganisms before they can be used as probiotics and/or food additives. In our study, all the enterococcal strains tested were sensitive to vancomycin, which is a positive phenotype for selecting these strains as potential probiotics since vancomycin is one of the most clinically relevant antibiotics.

In our study, several bacteria inhibiting strains were selected from both the skin and intestines of the European sea bream (*S. aurata*). Other studies also showed that the skin and gastrointestinal tract of various fish species contain lactic acid bacteria that produce antibacterial compounds able to inhibit the growth of several microorganisms (Ringo 1999; Spanggaard et al., 2001; Rengpipat et al., 2008; Vijayabaskar and Somasundaram, 2008; Ringo, 2008). The antimicrobial spectra on inhibition observed for the *Enterococcus* species included several genera, which indicates a broad spectrum of activity against Gram-positive but also against Gram-negative pathogenic and spoilage organisms.

The fact that these LAB Gram-positive bacteria showed great inhibitory activity towards Gram-negative pathogens is interesting because it is in contrast to the belief that the inhibitory spectrum of LAB is generally restricted to other Gram-positive bacteria (Abee et al., 1995). In agreement with our results, some LAB have been reported to inhibit Gram-negative fish pathogens (Gildberg and Mikkelsen, 1998; Joborn et al., 1997; Ringo, 2008; Robertson et al., 2000). Also, a number of earlier studies have also shown that several marine bacteria produce inhibitory substances that inhibit bacterial pathogens in aquaculture systems (Nogami and Maeda, 1992; Austin et al., 1995; Rengpipat et al., 1998; Gram et al., 1999; Chahad et al., 2007).

Many strains of enterococci, mainly *E. faecalis* and *E. faecium,* are known to produce a variety of bacteriocins active against several pathogenic bacteria, such as *Listeria monocytogenes, Staphylococcus aureus,* and *Vibrio* sp. (Ogier and Serror, 2008). Given their commensal status, enterococci are used as probiotics for humans or farm animals (Tannock and Cook, 2002; Sayyed et al., 2014). The use of such bacteria to inhibit pathogens by the release of antimicrobial substances is now gaining importance in fish farming as a better and more effective alternative to the use of antibiotics to manage the health of these organisms (Vijayan et al., 2006, Iman et al., 2014).

This research has confirmed the abundance of enterococci in European sea bream, both at the skin and intestinal levels and proves that many of the enterococci exhibit inhibitory activity against a number of pathogen and spoilage strains. The selected enterococcal strains described in this study are currently under characterisation to elucidate their potential use as probiotic

bacteria in aquaculture.

Conflict of interests

The authors did not declare any conflict of interest.

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Culturable bacterial diversity and hydrolytic enzymes from drass, a cold desert in India

Puja Gupta and Jyoti Vakhlu*

School of Biotechnology, University of Jammu, Jammu- 06, J & K, India.

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Bacterial diversity of composite soil sample of drass was explored and screened for various hydrolytic enzymes. About 600 bacterial strains were isolated using six different growth media, that is, R2A, nutrient agar, King's B media, tryptic soy agar, Luria-Bertani agar and minimal media (100 isolates picked randomly from each media). These bacterial isolates were further differentiated on the basis of colony/cell morphology analysis, pigmentation and growth patterns. The 99 selected strains were subjected to amplified ribosomal DNA restriction analysis and the representative isolates from each cluster were chosen for 16S rRNA gene sequencing. Phylogenetic analysis led to the identification of 40 bacteria, grouped into three major phyla, Proteobacteria, Actinobacteria and Firmicutes differentiated into 17 different genera. These representatives were also investigated for hydrolases at low temperature (4-30°C). All the isolates secreted one or the other hydrolytic enzyme, that is, esterase (90%), lipase (80%), protease (32.5%), amylase (20%), cellulase (17.5%). These results indicate that culturable bacteria in soil of Drass could serve as an ideal candidate region for enzyme bioprospecting.

Key words: Pigment, drass, cultivable bacteria, phylogenetic diversity, enzyme production, soil.

INTRODUCTION

Microorganisms in the cold environments have received increasing attention during the past decade as they play a major role in food chains and biogeochemical cycles of these environments (Margesin and Miteva, 2011). Diverse bacteria have been recovered from polar environments such as Arctic and Antarctic. However, diversity in polar regions differ from several high-altitude regions such as the Himalayan ranges due to seasonal variations in temperature that results in different physical and biochemical properties. Studies on non-polar environments particularly Himalayan region have been largely carried out on glaciers and snow samples (Pradhan et al., 2010; Shivaji et al., 2011). There are very few reports on bacterial diversity of Himalayan hilly terrains. Microbes inhabiting these cold environments are extensively prospected for unique adaptabilities of their enzymes (de Pascale et al., 2008). Cold adapted enzymes have high catalytic efficiency and unique specificity at low and moderate temperatures, signify-cantly at higher rate than the mesophilic counterparts (Gerday et al., 1997). These enzymes offer economic benefits through energy savings as they wipe out the requirement for expensive heating step. Due to their distinctive properties, these enzymes have also

*Corresponding author. E-mail: jyotivakhlu@gmail.com or jyotimetagenomic@gmail.com. Tel: 0941919-17624.

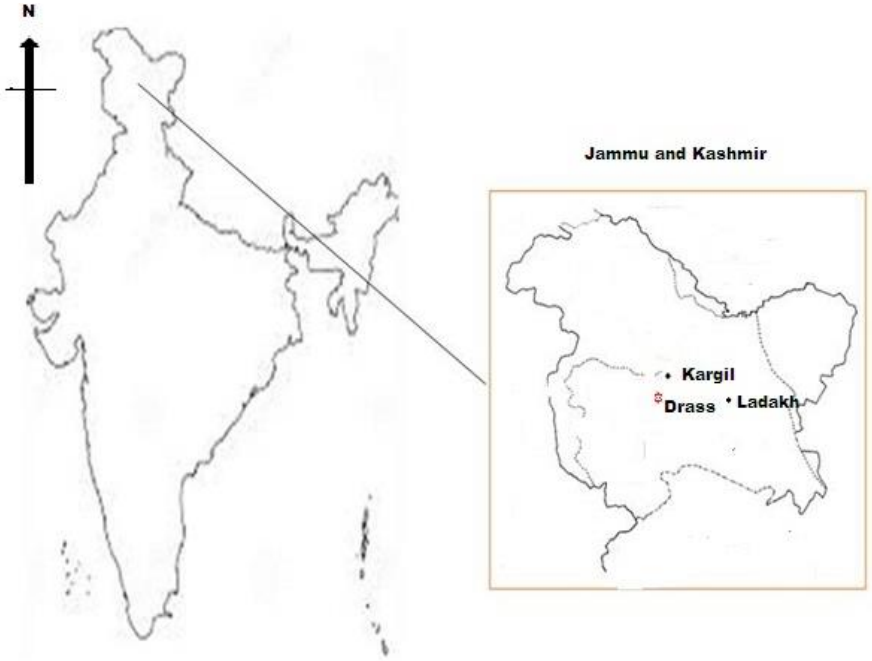

Figure 1. Outline map of Jammu Kashmir map showing the location of Drass (ladakh, J&K).

gained much attention regarding their potential for industrial and biotechnological applications, e.g cold adapted proteases are well suited in waste management in cold environments where the degradation capabilities of endogenous micro-flora are reduced due to low temperatures (Pulicherla et al., 2011). In industries, for dehairing of hides and skins using psychrophilic proteases not only save energy but also reduce the impacts of toxic chemicals used in de-hairing (Joshi and Satyanarayana, 2013). Amylases are one of the important industrial enzymes that have wide range of application such as food processing, fermentation and pharmaceutical industries. Cellulases are used in laundry detergents for exhibiting color brightness and removing the soil from cotton fibers, bio-polishing of fabric giving the finishing look of the product and producing stone washed look of denims (Aygan and Arikan, 2008; Sarvanan et al., 2013). Cellulases are gaining additional consideration in the enzyme market owing to their ability in the degradation of lignocellulosic biomass into biofuels and other products (Zhang et al., 2011). Lipases/esterases hold important position in the world enzyme market. The commercial use of lipases of cold origin is a billion dollar business. Psychrophilic lipases have attracted attention for synthesis of organic substances due to their inherent greater flexibility, whereas the activity of mesophilic and thermophilic enzymes are severely impaired by excess rigidity. These have great value for bioremediation and are widely used for degrading hydrocarbons present in contaminated soil (Aislabie et al., 2000; Paniker et al., 2006).

In the present study, an effort has been made to explore the bacterial diversity towards bioprospecting for hydrolytic enzymes from composite soil sample of Drass, located at 34.428152°N, 75.75118°E. It starts from the base of the Zojila pass (the Himalayan gateway to Ladakh), a trans-Himalayan region that separates the western Himalayan peaks from the Tibetan plateau (Figure 1). It is situated 60 km west of Kargil on the road to Srinagar with an average elevation of 3,280 m (10,764 ft) and experiences an altitude-influenced subarctic climate. The subarctic climate is characterized by long, usually very cold winters, and short, cool to mild summers. Winters start from mid-October and lasts in mid-May with temperature −22°C (-8°F) to as low as -45°C (-49°F) at the height of winter. Summers start in June and lasts till early September, with average temperatures near 15°C (59°F) and little precipitation. Annual precipitation is almost entirely concentrated in the months of December to May when Drass gets about 360 mm (14 inches) of snow.

MATERIALS AND METHODS

Collection of soil sample and sampling site

The soil samples (10) were collected from different regions of Drass mountains at 34.45°N, 75.77°E in North Himalayan range (J&K, Ladakh) during May 2010 and pooled into one composite sample. The soil was collected 1 cm deep into the earth by digging and collected in aseptic plastic bags (Shivaji et al., 2011). Hands, trowels were treated with 70% ethanol immediately before use. The samples were transported to the laboratory in ice and stored at -

20°C.

Enumeration and isolation of heterotrophic bacteria

One gram of sample was aseptically weighed and homogenized in 9 ml sterile physiological water (0.86% NaCl) by vortexing vigorously. Six different media namely R2A, Nutrient agar, King B agar, Tryptic soy agar, Luria-Bertani agar Minimal media were used to isolate bacteria by plating10^{-2} and 10^{-4} soil dilution with saline. All the growth media used in the present study were purchased from Himedia Pvt. Ltd India (Cat no. ≠ M962, M001, M1544, GM1151, M512, M290 respectively) and prepared according to the instructions given by the manufacture. The plates were incubated for 4-5 days in incubators at 4, 10, 20, 30°C temperature and the CFU/g of the soil was calculated.

Morphology and molecular identification

Preliminary taxonomic characteristics of the isolated bacteria were determined by colony morphology, pigment colour, growth pattern and biochemical analysis (Hamid et al., 2003). Pure cultures were cryopreserved in 50% glycerol at -80°C (New Brunswick, Effendorf). Genomic DNA was extracted by Hipura kit (Himedia, cat no.≠ MB505). Universal bacterial primers, namely Bac8f (AGTTTGATCCTGGCTCAG) & Univ529r (ACCGCGGCKGCTGGC) based on *Escherichia coli* positions, were used to amplify internal fragments of 16S rRNA gene that amplify ~500 bp (Fierer et al., 2007). PCR products were analyzed by electrophoresis on 1.5% agarose gel, followed by staining with ethidium bromide and visualization under UV light. The amplified PCR products were purified with a PCR product purification kit (Himedia cat no. ≠ MB512). ARDRA of the PCR products was done using restriction enzymes ALu I and Hha I to screen for duplicacy (Moreno et al., 2012). The unique bacterial isolates were sent for Sanger's DNA sequencing to Scigenome Labs Pvt. Ltd. (Cochin, India). For identification of closest relatives, sequences were compared to 16S sequences available in the GenBank (http://blast.ncbi.nlm.nih.gov) databases by BLASTn. The phylogenetic tree was constructed by MEGA 5 (http://www.megasoftware.net). The sequences that showed less than 98% homology with the reported sequences in the database were reamplified by bac8f (5-AGAGTTTGATCCTGGCTCAG-3) and 1492r (CGG TTA CCT TGT TAC GAC TT) corresponding to *E. coli* positions 8 to 27 and 1492 to 1509, respectively to amplify ~1500 bp region (Yong et al., 2011).

Diversity measures

OTUs at the 3% distance and Shannon-Wiener index (H) were calculated using Fastgroup11 tool (http://fastgroup.sdsu.edu/).

Screening for hydrolytic enzyme

Agar medium containing appropriate substrate and 1.5% agar (w/v) were inoculated with freshly grown cultures and incubated at 4, 10, 20, 30°C for 48 h. Different substrates for example 0.4% soluble starch (w/v), 0.4% (*w/v*) carboxymethylcellulose, 0.4% (w/v) tributyrin, olive oil (1%) and casein (0.4% w/v) were used for screening amylases, cellulases, esterase, lipases and proteases (Gangwar et al., 2009). For amylase and cellulase activity, incubated plates were developed by flooding the plates with iodine solution (1%) and washing with normal saline. For screening lipases, syringe filtrated olive oil (1%) and a florescent dye rhodamine B (0.001% w/v) was added to the autoclaved cooled growth medium with vigorous stirring. The plates containing bacterial cultures were observed for an orange fluorescence under UV light at 350 nm (Ranjitha et al., 2009).

RESULTS AND DISCUSSION

The present study is a first attempt to isolate and characterize the heterotrophic bacteria from soil of Drass, using different culturing conditions. Drass is the second coldest place in world after Siberia and its bacterial diversity (both cultivation dependent and independent) has not been unexplored so far. It is an established fact that cultivation based technique harvest only 1% of the bacteria and cultivation independent metagenomic techniques catalogue majority of the diversity. However, cultivation dependent conventional isolation techniques were employed as the aim of the present study was to isolate bacteria with hydrolytic activity that can be used commercially subsequently.

Bacterial isolation and characterization

Both oligotrophic and nutrient rich media were selected to obtain maximum cultivable bacteria. About 600 isolates were randomly selected (100 each from six different media: Nutrient agar, LB agar, King's B agar, TSA, Minimal media, R2A agar) used in the study. Since the average summer and winter temperature varies between 4-30°C, the bacteria were isolated within this temperature range. The growth pattern of individual bacterial culture were studied and placed into psychrophilic (4–20°C), psychrotrophic (4-30°C), and psychrotolerant mesophilic (4–37°C), mesophilic (25-40°C) groups (Sahay et al., 2013) (Table 1). Maximum bacterial load (including pigmented and non-pigmented 5.0± 0.07x 10^6 CFU/ml at 30°C CFU/ml was obtained using NA (Table 2) but maximum number of pigmented bacteria 2.9±0.17x10^6 were obtained with R2A media (Table 2). Pigment production was intense at 4°C and decreased with increase in incubation temperature which is in accordance with earlier studies on bacterial diversity of Puruogangri ice core (Zhang et al., 2008) and Himalayas (Venkatachalam et al., 2015). R2A is an oligotrophic medium and allows cultivation of many pigmented bacteria in particular that will not readily grow on fuller, complex organic media. R2A has been used to isolate bacteria from various cold environments e.g glaciers (Foght et al., 2004), marine surface waters (Agogue et al., 2005), ice cores (Zhang et al., 2008) and Antarctic soils (Dieser et al., 2010; Peeters et al., 2012). The pigments produced by these bacteria are reported to be carotenoids and has been co-related with cold adaptation of microorganisms by many workers (McDougald et al., 1998; Cho and Tiedje, 2000; Daniela et al., 2012; Mojib et al., 2013).

Diversity measures

Diversity indices were used to compare between the

Table 1. Taxonomic affiliations and phenotypic characterization of bacteria isolated from soils of Drass (J&K, Ladakh) determined by sequencing of 16S rRNA.

Close representative Organisms/ group	Phylum	Accession no.	Media used	Temperature range (°C)	Colony description
Pseudomonas vranovensis Dr1	Gammaproteobacteria	KF555604	King,s B	Psychrotolerant mesophilic	Pale yellow
Pseudomonas putida Dr2		JX978885	King,s B	Psychrotolerant mesophilic	Pale yellow, transparent
Pseudomonas fuscovaginae Dr5		JX978887	King,s B	Psychrotolerant mesophilic	Pale yellow, transparent
Pseudomonas stutzeri Dr12		KF555605	R2A	Psychrotolerant mesophilic	Yellow, wrinkled
Pseudomonas mandelii Dr13		JN088486	Kings B	Psychrotrophic	Yellow, smooth
Pseudomonas psychrotolerans Dr17		KF555606	R2A	Psychrotrophic	Yellow, wrinkled
Pseudomonas brenneri Dr29		KF555610	Kings B	Psychrotrophic	Yellow
Pseudomonas frederiksbergensis Dr27		KF555608	LB	Psychrotrophic	Pale yellowish
Acinetobacter calcoaceticus Dr4	Alphaproteobacterria	JX978886	LB	Psychrotolerant mesophilic	White, slimy
Acinetobacter spp. Dr11		JX978891	LB	Psychrotrophic	cream
Acinetobacter radioresistens Dr25		JX978884	NA	Psychrotolerant mesophilic	cream
Serratia proteamaculans Dr7		JX978888	LB	Psychrotolerant mesophilic	White, slimy
Pantoea agglomerans Dr31		KF555611	R2A	Psychrotolerant mesophilic	Dark yellow
Pantoea agglomerans Dr46		KM188063	R2A	Mesophilic	Yellow
Paracoccus marcusii Dr32		KF555612	R2A	Psychrotolerant mesophilic	orange
Bacillus safensis Dr6	Firmicutes	KF682429	LB	Mesophilic	White Opaque
Bacillus cereus Dr8		JX978889	LB	Mesophilic	Cream slimy
Bacillus atrophaeus Dr14		JX978892	NA	Mesophilic	Brownish black, rough
Bacillus simplex Dr18		JN088488	NA	Psychrotolerant mesophilic	Cream slimy
Bacillus spp. Dr22		JN088491	NA	Psychrotrophic	Cream
Bacillus vallismortis Dr38		KF555618	LB	Mesophilic	Black
Bacillus thuringiensis Dr45		KF555624	NA	Psychrotrophic	Off-white
Staphylococcus equorum Dr34		KF555614	LB	Psychrotolerant mesophilic	White
Sporosarcina psychrophila Dr35		KF555615	MM	Psychrotolerant	Beige, shiny
Sporosarcina psychrophila Dr41		KF555620	R2A	Psychrotrophic	Brownish
Exiguobacterium sibiricum Dr19		JX978893	MM	Psychrotrophic	Orange, smooth
Exiguobacterium undae Dr28		KF555609	R2A	Psychrotrophic	Light orange
Planomicrobium koreense Dr24		JX978895	TSA	Psychrotolerant mesophilic	Orange
Arthrobacter agilis Dr16	Actinobacteria	JX978896	R2A	Psychrotrophic	Rose red, smooth
Arthrobacter crystallopoietes Dr37		KF555617	R2A	Psychrotrophic	Light yellow
Mycetocola reblochoni Dr23		HE774268.1	R2A	Psychrotrophic	Yellow, smooth
Mycetocola reblochoni (Dr42)		KF555621	TSA	Psychrotolerant mesophilic	Light yellow
Kocuria Polaris (Dr20)		KF682428	R2A	Psychrotrophic	Red and smooth
Kocuria rosea (Dr33)		KF555613	R2A	Psychrotrophic	Dark pink

Table 1. Contd.

Rhodococcus erythropolis (Dr10)	JX978890	MM	Psychrotrophic	Pale yellow, slimy
Rhodococcus qingshengii (Dr21)	JX978894	R2A	Psychrotolerant mesophilic	Light pink
Rhodococcus erythropolis (Dr36)	KF555616	MM	Psychrotrophic	White, very slimy
Citricoccus alkalitolerans (Dr40)	KF555619	R2A	Psychrotolerant mesophilic	Light yellow
Dietzia schimae (Dr43)	KF555622	TSA	Psychrotolerant mesophilic	reddish orange
Micrococcus luteus (Dr44)	KF555623	NA	Mesophilic	Yellow

NA, Nutrient agar; LB, Luria-Bertani agar; MM, minimal media; TSA, tryptic soy agar.

Table 2. Bacteria load (pigmented and non-pigmented) on six different media at different temperatures.

Growth media	C.F.U at 4°C		C.F.U at 10°C		C.F.U at 20°C		C.F.U at 30°C	
	(P)	(NP)	(P)	(NP)	(P)	(NP)	(P)	(NP)
R2A	0.5±0.15 x10^6	0.2±0.25 x10^6	1.2±0.05 x10^6	0.8± 0.02x10^6	2±0.09 x10^6	0.8± 0.02 x10^6	2.9±0.17x10^6	1.2±0.0 3x10^6
NA	0.07± 0.02 x10^6	0.13 ± 0.02 x10^6	0.1±0.01x10^6	0.7±0.2x10^6	0.1± 0.01 x10^6	0.9 ± 0.03x10^6	1.0± 0.01x 10^6	4±0.06 x10^6
LB	0.18±.10 x10^6	0.22±0.05x10^6	0.4± 0.02x10^6	1.6±0.06 x10^6	0.6± 0.01x10^6	2.4±0.1x10^6	0.9± 0.07x10^6	3±0.10 x10^6
Kings B	0.3±.10x10^6	0.1±0.07 x10^6	0.4± 0.16x10^6	0.2 ±0.13x10^6	0.6 ±0.3 x10^6	0.2± 0.05 x10^6	1.4±0.05x10^6	0.8±0.02 x10^6
TSA	0.2± 0.10x10^6	0.3± 0.12x10^6	0.3± 0.02x10^6	0.6±0.03x10^6	0.5±0.02x10^6	1.5±0.05 x10^6	1.0 ±0.13x10^6	2.3±0.10 x10^6
MM	0.2± 0.09 x10^6	0.1± 0.05 x10^6	0.4 ±0.15x10^6	0.1± 0.05x10^6	0.6± 0.3 x10^6	0.2±0.1 x10^6	1.8± 0.040 x10^6	0.4±0.005x10^6

C.F.U counted as cells/ml; Experiments were conducted in triplicates and the data are expressed as mean± SD.

communities obtained by using different media. More community complexity was found using R2A media (Figure 2 and Table 3). Overall Shannon-Wiener index (H) was 3.2, that is in accordance with previous reports from Himalayan bacterial diversity (Pradhan et al., 2010; Shivaji et al., 2011, Yadav et al., 2014).

Phylogenetic analysis of 16S rDNA sequences of isolates

Bacterial isolates were screened for duplicacy by colony/cell morphology analysis, pigmentation, conventional biochemical tests that narrowed the 600 isolates into 99 isolates. These selected isolates were subjected to 16S rRNA gene amplification followed by restriction digestion with Alu I and Hha I. On the basis of ARDRA profiling, representative isolate from each cluster were sequenced and the nucleotide sequences were deposited in the NCBI GenBank database (Accession numbers: JX978884-JX978891, JX978892-JX978896, JN088486, JN088488, JN088491, KF555604-KF555606, KF555608-KF555624, KF682428, KF682429 and HE774268, KM188063). The nearest phylogenetic neighbor of all the 40 representative isolates were identified through BLAST analysis of the 16S rRNA gene sequences against nucleotide database available in the National Centre for Biotechnology Information (NCBI) (Table 1).

Drass isolates represented both Gram-positive and negative heterotrophic bacteria belonging to three major phylogenetic groups organized into three clusters, Proteobacteria (37.5%), Firmicutes (32.5%) and Actinobacteria (30%) (Figure 3). Proteobacteria dominates (37.5%) the culturable bacterial diversity of Drass with Gammaproteobacteria (35%) as the dominant class represented by genera *Pseudomonas, Acinetobacter, Serratia* and *Pantoea.* Pseudomonads represented the dominant genera among Gammaproteobacterium. Alphaproteobacteria is however represented by single genera, that is, Paracoccus (Dr32) (Figure 3). The results are in accordance with the previous studies on Himalayan that reports Firmicutes, Actinobacteria and Proteobacteria as the most common phylum (Shivaji et al., 2011).

Bacterial isolates showed 99% similarity with the reference sequences in the Genbank except for Dr 46 that showed 96% similarity with *Pantoea agglomerans* (Figure 4). DNA-DNA hybridization

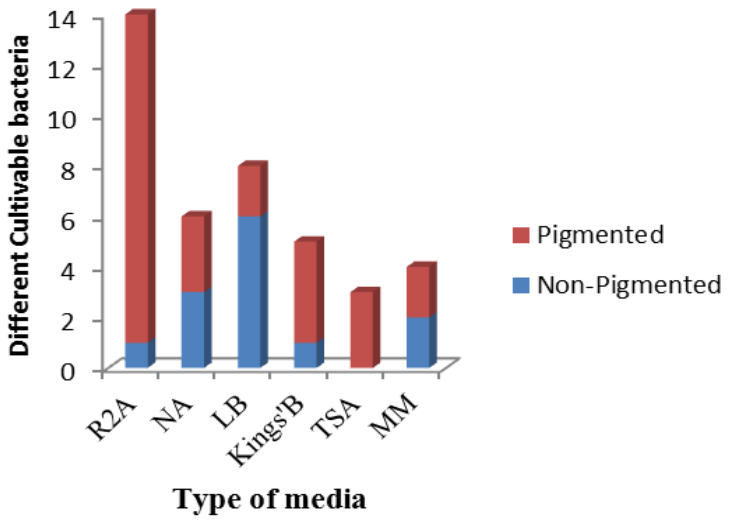

Figure 2. Diversity of pigmented and non-pigmented bacteria on six different media. NA, Nutrient agar; LB, Luria-Bertani agar; MM, minimal media; TSA, tryptic soy agar.

Table 3. OTUs and Shannon-Wiener index represented by bacteria on different growth media.

Growth media	OTUs	Shannon-Wiener index
R2A	14	2.54
LB	8	2.07
NA	6	1.79
Kings B	5	1.60
Minimal media	4	1.38
TSA	3	1.09

NA, Nutrient agar; LB, Luria-Bertani agar; MM, minimal media;TSA, tryptic soy agar.

will be carried with close relatives to confirm and publish as novel species. The bacteria isolated and characterized from Drass soil have been reported from other cold environments also. The genera *Arthrobacter, Bacillus, Sporosarcina, Rhodococcus, Pseudomonas* were reported in the culturable bacterial diversity of Pindari glacier (Shivaji et al., 2011). The genera *Acinetobacter, Bacillus, Pseudomonas* were reported in the culturable bacterial diversity of Kafni glacier (Srinivas et al., 2011). The genus *Arthrobacter* is the dominant bacteria in Qinghai-Tibet Plateau permafrost (Zhang et al., 2007), *Brevibacterium* and *Acinetobacter* are present in abundance in Dry Valley soils of Antarctica (Cary et al., 2010), *Planomicrobium, Mycetocola, Rhodococcus, Sporosarcina* have been reported from Himalayan soils in India and Nepal (Venkatachalam et al., 2015) and an Arctic glacier (Reddy et al., 2009). *Exiguobacterium* (Gram positive and facultatively anaerobic) have been repeatedly isolated from ancient Siberian permafrost (Rodrigues et al., 2009). Members of genera *Exiguobacterium* are adapted to long-term freezing at temperatures as low as -12°C where intracellular water is not frozen and grow at subzero temperatures, displaying several feature of psychrophiles, such as membranes composition. Genus *Pantoea* (Selvakumar et al., 2008; Venkatachalam et al., 201**5**), *Dietzia* (Mayilraj et al., 2006), *Staphylococcus* and *Citricoccus* (Yadav et al., 2015) have been reported from Indian Himalayas. Members of the genus *Paracoccus* have been reported from Qinghai-Tibet Plateau permafrost (Zhu et al., 2013).

Extracellular hydrolytic enzyme activity

Cold-active enzymes from microbial sources have potential applications in biotechnology, agriculture and medicine (Feller, 2007; Tropeano et al., 2012; Moreno et al., 2013). The representative isolates were screened for their extracellular hydrolytic enzyme activity viz., esterase, lipases, protease, amylase and cellulose.

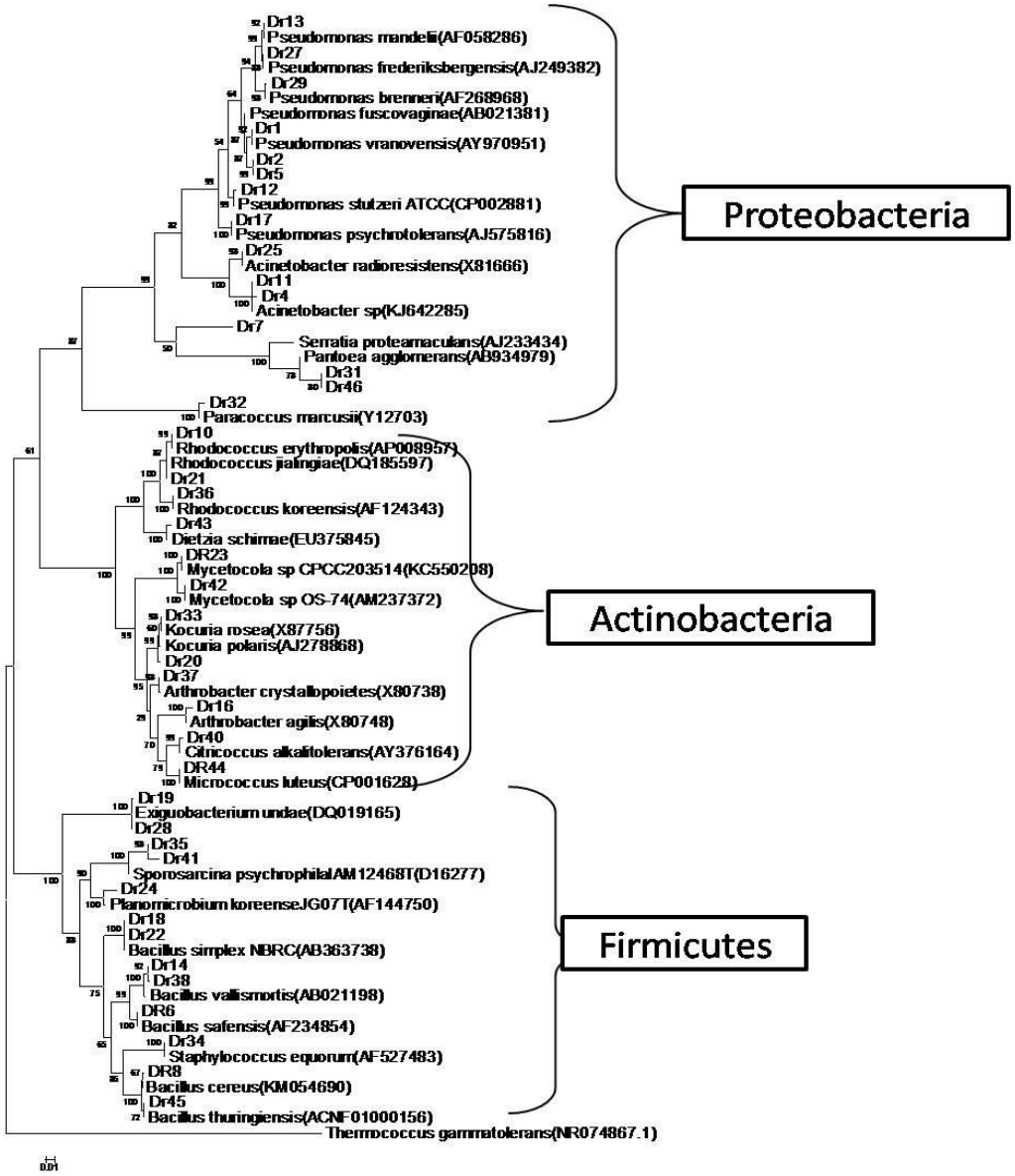

Figure 3. Maximiun likelihood phylogenetic tree of 16S rRNA gene from a soil sample of Drass (J&K, Ladakh). Bar, 0.01 substitutions per nucleotide position. *Thermococcus gammatolerans* was used as an outgroup

Interestingly, 90% of the isolates were esterase producers and out of them 80% were lipase producers, 32.5% were protease producers, 20% were amylase producers and 17.5% are cellulose producers. The comparative profile of the hydrolytic enzymes produced by bacterial isolates of Drass has been represented (Figure 5). *Arthrobacter agilis* Dr16 and *Kocuria Polaris* Dr20 were the best esterase producers and the enzymes produced were active at low temperatures (10°C). *Mycetocola reblochoni* Dr23 and *Planomicrobium koreense* Dr24 though best protease producers, do not degrade skimmed milk below 20°C. *Bacillus cereus* Dr8 and *Acinetobacter radioresistens* Dr25 were multiple hydrolases producer at low temperatures (10°C). The results clearly indicated that these enzymes can be characterized for exploitation at industrial level. Gangwar and coworkers 2009 worked on the bacterial diversity isolated from soil samples from the western Himalayas, India and reported that 62% of the bacterial isolates produced lipase followed by protease, 54%, Amylase 28% and only 11% have cellulase activity (Gangwar et al., 2009). *Pseudomonas, Bacillus, Arthrobacter, Exiguobacterium, Mycetecola, Pantoea, Acinetobacter* and *Serratia* have been identified as hydrolytic enzyme producer in previous study on Himalayas (Salwan et al., 2010; VenKatachalam et al., 201**5**). Although, in addition other genera especially *Kocuria, Rhodococcus* and *Planomicrobium* isolated in our study also showed hydrolytic enzyme activity.

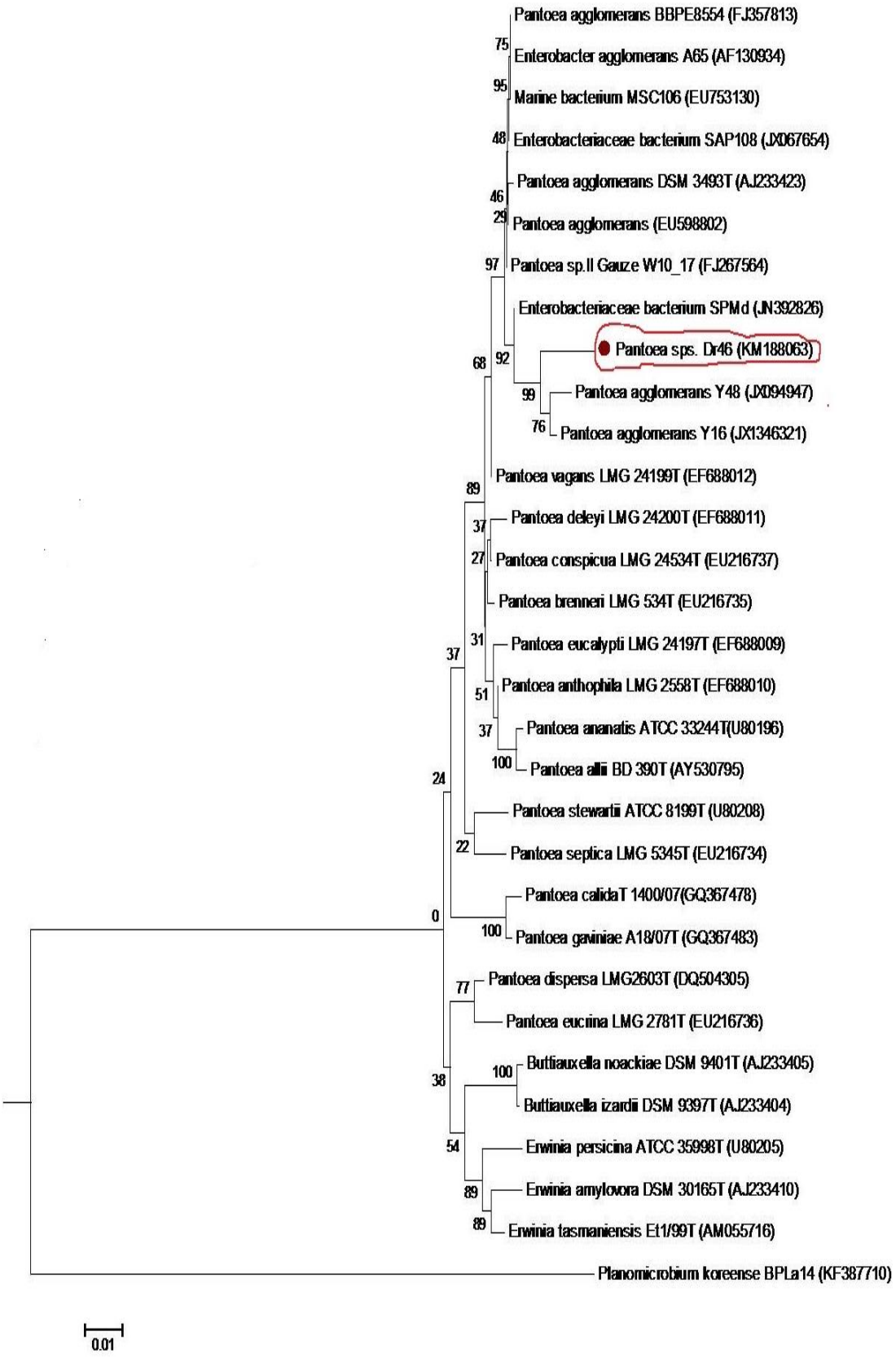

Figure 4. Neighbour joining tree based 16S rRNA sequences showing the positions of strain Dr46 *Pantoea* sp. and represenatives of some other taxa. Bar, 0.01 substitutions per nucleotide position. *Planomicrobium Koreense* BPLa14 was used as an outgroup.

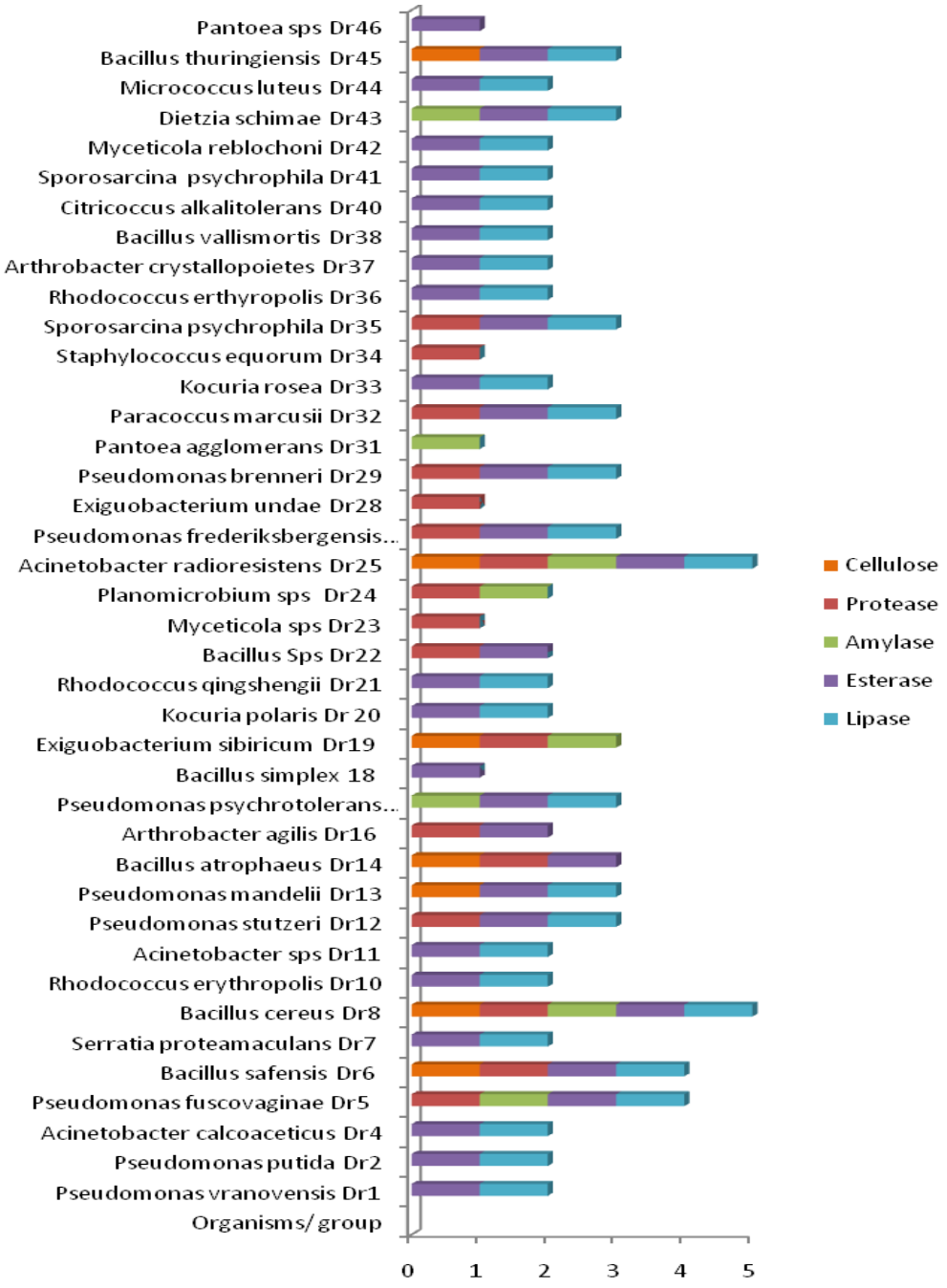

Figure 5. Enzyme profile of bacterial isolates from Drass (J&K, Ladakh).

Conclusion

Though earth is dominated by cold habitats but most of the diversity studies and bioprospecting has been done either on thermophiilic/thermotolerent or mesophillic bacteria. Recently, focus on exploring cold environments for diversity and bioprospecting has increased due to energy concerns and ecological reasons. Drass, located in the Western Himalayas, the second coldest place in India was found to be a rich source of novel bacteria and their produce. Interestingly Dr46 has low percentage similarity with the reference strain *Pantoea* and could probably be new species. The low percentage of probable novel bacteria isolated despite using six media and starting from 600 isolates suggests that more media formulation need to be tried and larger population needs to be characterized. Further screening for hydrolytic enzyme activity resulted in screening of some of the

multiple hydrolase producers for industrial use and not merely cataloguing them as is possible in the cultivation independent approach.

Conflict of interest

The authors declare that there is no conflict of interest.

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Full Length Research Paper

Isolation and optimization of amylase producing bacteria and actinomycetes from soil samples of Maraki and Tewedros campus, University of Gondar, North West Ethiopia

Gebreselema Gebreyohannes

Department of Biology, College of Natural and Computational Sciences, University of Gondar, P. O. Box 196, Gondar, Ethiopia.

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The objective of the present study was to isolate, identify and optimize potential amylase producing bacteria and actinomycetes from soil samples. The soil samples were collected from Maraki and Tewedros campus, University of Gondar. Isolation was done by serial dilution and spread plate method. Primary screening of amylolytic activity of the isolates was performed by starch agar plate method. The submerged state fermentation method followed for the production of amylase by the optimization of temperature, pH, fermentation time and substrate concentration. From the soil samples, 18 isolates were identified and subjected to primary screening for amylolytic activity. Of which, five isolates were observed with maximum amylolytic activity during the primary screening. During the submerged state fermentation, maximum amylase activity was observed at 48 h and then declined. The optimum temperature observed for maximum amylase activity of *Bacillus* was 40°C and *Streptomyces* at 37°C. The highest amylase activity was observed at neutral pH and 4% of starch concentration. The colony morphology, Gram reaction, biochemical tests and Bergey's manual of determinative bacteriology confirm the promising isolates belong to the genus *Bacillus* and *Streptomyces.* This preliminary study could provide base line information for the discovery of novel microbes from the natural resources for the production of amylase which will be used for multipurpose.

Key words: Amylase, isolation, optimization, submerged state fermentation.

INTRODUCTION

Amylase is an enzyme obtained from the microbes has been used by many industries as a source for production of foods and beverages. With the utilization of microorganisms it is possible to produce large scale and also easily manipulated for desired products (Sumrin et al., 2011). In general, enzymes produced from fungal and bacterial sources have many applications in industries (Aiyer, 2005). In addition, recent advancement in

*Corresponding author. E-mail: ggebreselema@yahoo.com.

biotechnological tool, utilization of amylase has widened in clinical research, medical chemistry and starch analytical chemistry. Earlier literatures highlighted that bacterial strains from the genus *Bacillus*, *Pseudomonas* and *Clostridium* and from the genus *Streptomyces* have been used to synthesize amylase (Kafilzadeh et al., 2012; Oyeleke et al., 2010). Multi-potential application and demand pave the way for increasing indigenous amylase production and searching for more efficient processes (Hmidet et al., 2009). In Ethiopia, there is no much work done in this area of research. The country has several undisturbed natural soil habitat. In this research work soil samples were collected from various locations of Maraki and Tewodros campus since these areas are abundant in plant biodiversity and soil types. Therefore, to contribute new knowledge in scientific world amylase producing microorganisms were isolated, identified, optimized and reported for the first time from this study area.

MATERIALS AND METHODS

Sample collection

Soil sample were collected from five locations in Maraki and Tewedros campus, University of Gondar, Ethiopia. The study area is located in the latitude and longitude of 12° 35' 21" N / 37° 26' 39" E. From the selected area, 100 g of top soil samples was collected after careful removal of debris in the collection site. The soil samples were collected by using a sterile spatula, kept in the polyethylene bag and transported to the microbiology laboratory for further analysis. Stock soil samples were stored at 4°C in a refrigerator for subsequent analysis.

Screening of potential amylase producing bacteria and actinomycetes by using starch hydrolysis test

Ten gram of soil sample was suspended in 90 mL of sterile saline water in a conical flask and mixed by vortex mixer. From this 10 mL of the diluted suspension was transferred into three conical flasks containing 90 mL of sterile saline water serially. From each conical flasks, 0.1 mL was transferred into starch agar plates (meat extract 3 g/L; peptic digest of animal tissue 5 g/L; soluble starch 2 g/L; agar 15 g/L; pH 7.2 ± 0.1) in triplicate. Then, the sample was distributed evenly by using L-shaped glass rod and incubated at 37ºC for 24 h. After incubation period, colonies were further sub-cultured on the respective medium to obtain pure isolates and maintained at 4°C in a refrigerator for further investigation. The isolates were screened for amylolytic activity by streaking on the starch agar plates and incubated at 37°C for 24 h. Iodine solution was flooded on the starch agar plates for 30 s after 24 h incubation. Presence of clear zone around the growth of isolates were considered as amylase producers and sub-cultured on starch agar slants for further investigation.

Amylase production by using submerged state fermentation

A mineral broth medium (peptone 6 g/L; $MgSO_4$ 0.5 g/L; KCl 0.5 g/L and starch 1 g/L) was prepared. From the broth medium, 90 mL was transferred into 150 mL capacity Erlenmeyer flasks and sterilized at 121°C for 15 min. A loopful of inoculum was transferred into five test tubes having a 10 mL of sterile nutrient broth. The test tubes were incubated at 37°C for 24 h until the visible turbidity and density becomes equal to 0.5 McFarland standards ($1x10^8$ CFU/mL). Then after, 2 mL suspension of the isolates was taken from overnight cultures of test tube and inoculated into 90 mL of flasks and incubated in a water bath by adjusting the temperature at 25, 30, 35, 37, and 40°C for 24, 48, 72 and 96 h under the rotary shaker by the speed of 150 rpm. Finally, the fermented culture was poured into centrifuge tubes, spin for 20 min at 5000 rpm and extracted by decantation method (Aiyer, 2005).

Effect of substrate concentration and fermentation time

The effect of substrate concentration was determined by using different concentrations of starch (1.0, 2.0, 3.0, 4.0 and 5.0%) in the amylase production medium. The effect of fermentation time was also determined by incubating the amylase production medium at different fermentation time (24, 48, 72 and 96 h).

Effect of pH and temperature

The effect of pH and temperature on amylase activity was confirmed by adjusting the pH value of the fermentation medium at 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and the temperature at 25, 30, 35, 37 and 40°C respectively.

Characterization and identification of amylase producing isolates

Cultural, morphological and biochemical characterization of isolates

The isolates were observed macroscopically and microscopically to characterize the colony morphology and gram reaction respectively. In addition, isolates were also characterized biochemically by different biochemical tests such as Simmons citrate, Urease, Methyl Red/Voges Proskauer (MR/VP) and Starch hydrolysis tests.

RESULTS AND DISCUSSION

Isolation and primary screening of amylase producing bacteria and actinomycetes

A total of 18 isolates were obtained from the collected soil samples and coded as Sp1-Sp18 based on their maximum clear zone respectively. Of the 18 isolates, five isolates showed higher clear zone after flooding with iodine solution (Table 1) and this result agreed with the report of Hmidet et al. (2009) and only a few selected strains of bacteria from soil sample were obtained. According to Bergey's Manual of Determinative Bacteriology, isolates were grouped into two genera; namely, genus *Bacillus* (Sp1, Sp3, Sp4 and Sp5) and genus *Streptomyces* (Sp2). As per the primary screening of the isolates, these two genera could be potential candidates for several industrial applications which were agreed by the report of Ashwini et al. (2011).

The five isolates were characterized by cultural and microscopic methods to differentiate their respective genera. Most of the isolates have shown a regular form,

Table 1. Isolates and their clear zone on starch agar plates during primary screening.

Isolates	Clear zone (mm)	Isolates	Clear zone (mm)
Sp1*	22 ± 0.20	Sp10	8 ± 0.01
Sp2*	20 ± 0.50	Sp11	7 ± 0.90
Sp3*	19 ± 0.20	Sp12	6 ± 0.55
Sp4*	18 ± 0.60	Sp13	6 ± 0.32
Sp5*	18 ± 0.10	Sp14	5 ± 0.45
SP6	11 ± 0.40	Sp15	5 ± 0.31
Sp7	10 ± 0.70	Sp16	5 ± 0.56
SP8	10 ± 0.90	Sp17	4 ± 0.11
Sp9	10 ± 1.00	Sp18	3 ± 0.22

Mean ± Standard deviation of triplicate determination for primary screening, *Isolates selected for amylase production.

Table 2. Cultural and microscopic characteristics of the five isolates.

Characteristics	Isolates				
	Sp1	Sp2	Sp3	Sp4	Sp5
Form	Regular	Irregular	Regular	Regular	Regular
Color	Creamy	Rough whitish	Creamy	Creamy	Creamy
Gram staining	Positive	Positive	Positive	Positive	Positive
Shape	Rod	Filamentous	Rod	Rod	Rod

Table 3. Biochemical characteristics of isolates.

Biochemical characters	Isolates				
	Sp1	Sp2	Sp3	Sp4	Sp5
Starch hydrolysis test	+	+	+	+	+
Urease test	+	+	+	+	+
Simon's Citrate test	+	-	-	-	+
Methyl Red/Voges Proskauer test	+/+	+/+	+/+	+/+	+ /+
Indole test	+	+	+	+	+

+ Positive, - Negative.

creamy color and rod shape of colony morphology (Table 2).

Biochemical characterization

Based on the biochemical tests, all the isolates showed positive results of starch hydrolysis, Urease, MR/VP and indole tests (Table 3).

Effect of fermentation time on amylase activity

All the isolates were showed maximum amylase activity at 48 h of submerged fermentation time and then declined (Figure 1). Similar findings were also observed on *Bacillus subtilis* and *Bacillus* sp. DLB9 (Shyam et al., 2013). The reason for amylase activity decrement after 48 h might be due to the suppression and accumulation of other by-products in the fermentation medium and also depletion of nutrients as reported by other studies (Haq et al., 2010).

Effect of starch concentration on amylase activity

In general, amylase activity was increased with the increment of starch concentration from 1 to 4%. In this study, highest amylase activity was observed at 4% starch concentration (Figure 2). If the starch concentration

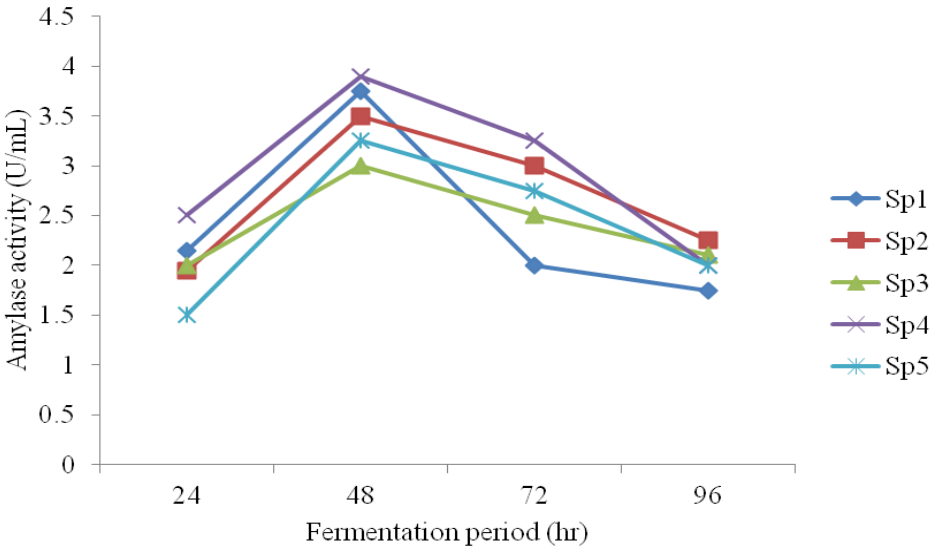

Figure 1. Effect of fermentation time on amylase activity.

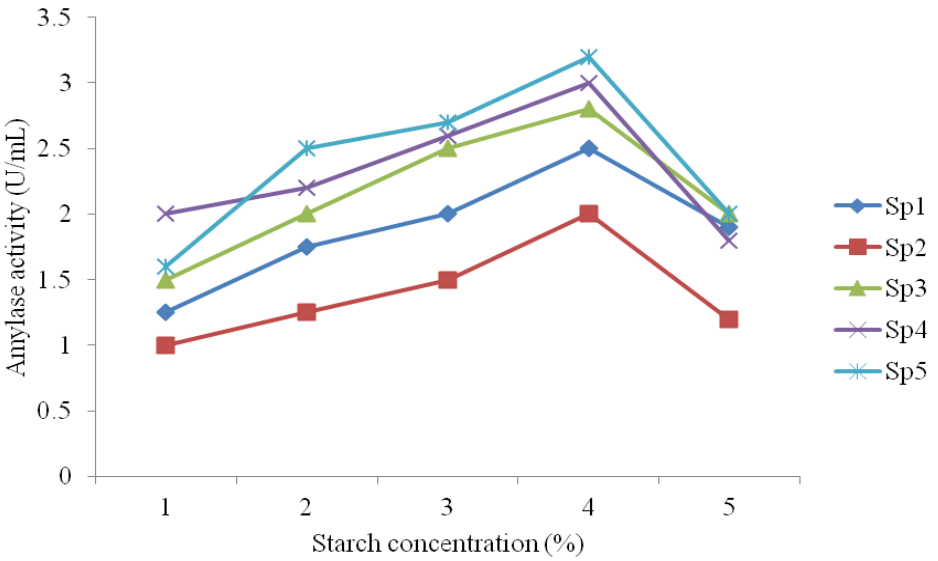

Figure 2. Effect of starch concentration on amylase activity.

goes beyond 4% amylase activity was declined. This might be associated with metabolizing capacity of the isolates within the short period of time when the starch concentration was increased. The present findings are in corroborating with the report of earlier findings on amylase activity obtained from *Bacillus* species (Oyeleke and Oduwole, 2009).

Effect of pH on amylase activity

In this study, highest amylase activity was observed at neutral pH. These results was also in agreement with the previous report for amylase activity of *Bacillus* strains such as *B. thermooleovorans* NP54, *B. coagulans*, *B. licheniformis*, and *B. subtilis* JS-2004 within the range of 6-7 pH (Gupta et al., 2010; Mendu et al., 2005; Adeyanju et al., 2007; Mrudula and Kokila, 2009). This implies that capability of the amylase activity within the neutral pH might be due to the fact that the isolates were inactive in the acidic or alkali medium. Different microorganisms have different optimum pH; if any variation on pH value results in poor microbial growth and amylase activity (Lonsane and Ramesh, 2009; Pandey et al., 2000).

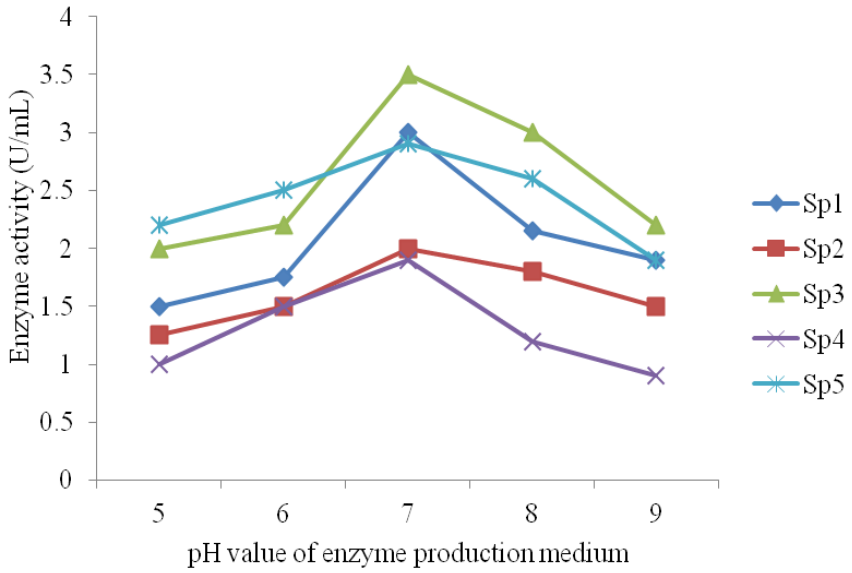

Figure 3. Effect of pH on amylase activity of the five isolates.

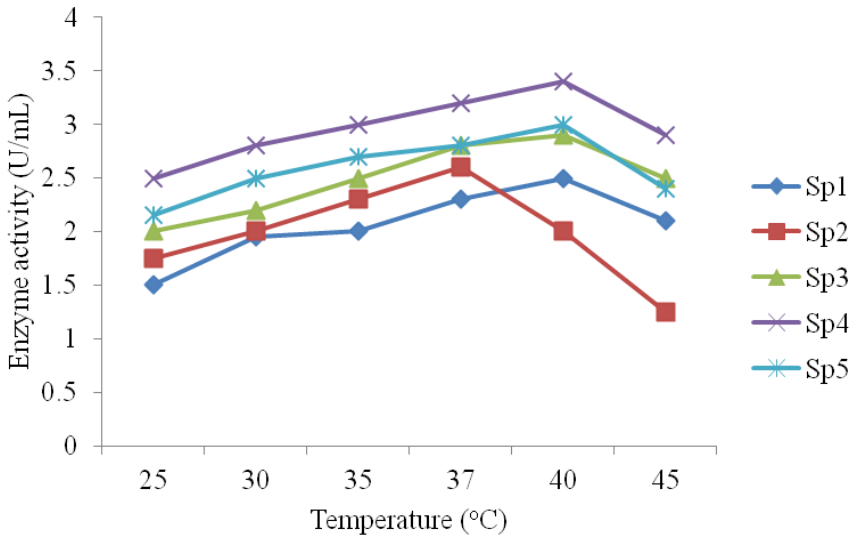

Figure 4. Effect of temperature on amylase activity.

(Figure 3).

Effect of temperature on amylase activity

Temperature is one of the environmental factors for amylase production which is usually varied from one organism to another. For example, *Bacillus amyloliquefaciens, B. subtilis, B. licheniformis* and *B. stearothermophilus* are some of the commonly used *Bacillus* sp. reported to produce amylase at 37-60°C (Asgher et al., 2007). In the present study, maximum amylase activity was observed at 40°C (Figure 4). The result also showed a positive correlation between the amylase activity and the incubation temperature up to 40°C, followed by a gradual decrease. At higher temperature, bacterial growth gets suppressed and consequently amylase activity was also inhibited (Oyeleke and Oduwole, 2009). The isolates grown well and revealed high amylase activity in the temperature ranged from 35 to 40°C. However, maximum amylase activity of *Bacillus* was 40°C and *Streptomyces* at 37°C. Mishra and Behera (2008) also reported that most of the bacterial isolates were produced and showed amylase

activity at elevated temperature in particular amylase activity of *Bacillus* species at the range of 40-45°C.

Conclusion

Based on the present findings, it is concluded that the soil is a potential source for amylase producing microorganisms, which could be exploited for the production of important industrial amylase. The results also showed that there was appreciable high amylase production from the isolates under optimized conditions of fermentation time, temperature, pH and starch concentration. *Bacillus* was found to be most frequently occurring amylolytic bacteria followed by *Streptomyces*.

Conflict of interests

The author declared that there is no conflict of interest.

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Short Communication

Action of proteases of the nematophagous fungi *Pochonia chlamydosporia* on *Ascaris suum* eggs of collared peccary (*Pecari tajacu*)

Filippe Elias de Freitas Soares[1]*, José Humberto de Queiroz[1], Jackson Victor de Araújo[2], Maria Gorete Ramos Rodrigues[3], Alexandre de Oliveira Tavela[2], Anderson Rocha Aguiar[3], Tracy Lacerda[3], Carolina Magri Ferraz[3], Maria Cristina Valdetaro Rangel[3], Thiago Senna[3], Andreia Luíza Araújo[3], Tarcízio de Paula Rego[2], Caio Colodette Sena[3] and Fabio Ribeiro Braga[3]

[1]Departamento de Bioquímica e Biologia Molecular. Universidade Federal de Viçosa, Viçosa-MG. Cep: 3657000, Brasil.
[2]Departamento de Veterinária, Universidade Federal de Viçosa, Viçosa-MG. Cep: 3657000, Brasil.
[3]Universidade Vila-Velha, Vila Velha, Espirito Santo. Cep: 29102206, Brasil.

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Among the parasites of domestic and wild swine, *Ascaris suum* stands out; a nematode that can lead to growth retardation and reduction in weight gain due to its action, especially in young animals. The objective of this study was to test the ovicidal action of proteases from *Pochonia chlamydosporia* (VC4) on *A. suum* eggs in an assay with Petri dishes. The fungus *P. chlamydosporia* (VC4) was grown in Erlenmeyers flasks with 50 ml of liquid minimal media supplemented with 0.2% gelatin for production of enzymes. In the present assay, 500 eggs were poured into Petri dishes of 4.5 cm in diameter and 5 ml of VC4 proteases were added in each Petri dish and incubated at 26°C in the dark for 14 days. After this period, the number of embryonated and destroyed *A. suum* eggs present in each plate from treated and control groups was counted. Significant difference (p <0.01) was found between the number of eggs from treated group compared to the control group. At the end of the experiment, the proteases of *P. chlamydosporia* (VC4) demonstrated efficacy in reducing embryonated eggs on the plates of the treated group (78.7%) compared to the control group (83.7%). The results presented in this study demonstrate that proteases of *P. chlamydosporia* (VC4) were effective in the destruction of *A. suum* eggs and therefore could be used as biological control of this nematode.

Key words: Nematophagous fungi, *Pochonia chlamydosporia*, protease, *Pecari tajacu*, *Ascaris suum*.

INTRODUCTION

The commercial breeding of wild animals in several countries has been identified as an important source of protein used to the livelihoods of the poorest people living in the countryside. However, there are some obstacles

*Corresponding author. E-mail: filippeufv@yahoo.com.br. Fax: +55 (31) 3899-3048.

for the commercial production of these animals, and, among these, the gastrointestinal nematodiosis, that deserve attention (Bonuti et al., 2002). Among the parasites of domestic and wild swine, *Ascaris suum* stands out, a nematode that can lead to growth retardation and reduction in weight gain due to its action, especially in young animals (Urquhart et al., 1998).

Worm control in animals is carried out with the use of anthelmintic drugs, however, these drugs may not be fully effective mainly due to the parasitic resistance (Bowman et al., 2006). Thus, alternative measures that may help to decrease the continued use of a same class of anthelmintic, as well as the use of doses higher than recommended, are required, and among these alternatives is the biological control conducted with natural antagonists of nematodes, with emphasis on the nematophagous fungi (Braga and Araújo, 2014). In the environment, these fungi are biologically very important because they play a role in the recycling of carbon, nitrogen and other elements that originate from the degradation of nematodes (Braga et al., 2007).

Nematophagous fungi are the major natural antagonists of nematodes in the environment. They are divided into predators, endoparasites and ovicides. In the group of ovicidal fungi, the species *Pochonia chlamydosporia* stands out (Araújo et al., 2008; Braga and Araújo, 2014). These fungi secrete extracellular enzymes from the class of proteases, which develop an important role in infection and destruction (ovicidal activity) of eggs of the nematodes (Yang et al., 2013; Khan et al., 2004). On the other hand, the ovicidal action of *P. chlamydosporia* (VC4) and its proteases has been successfully tested against eggs from various genera of helminth under laboratory conditions (Braga et al., 2008a, b, 2009a; Soares et al., 2014). However, this isolate had never their proteases tested on eggs of *A. suum* of a wild swine, as *Pecari tajacu*.

The objective of this study was to test the action of proteases of *P. chlamydosporia* (VC4) on eggs of *A. suum*.

MATERIALS AND METHODS

Fungus

One isolate of ovicidal fungus *P. chlamydosporia* (VC4) from mycology collection of the Parasitology Laboratory of the Department of the Federal University of Viçosa Veterinary, Minas Gerais, Brazil was maintained in test tubes at 4°C containing 2% corn-meal-agar (2% CMA) in the dark for 10 days.

Culture dishes of 4 mm in diameter were extracted from fungal cultures maintained in test tubes containing 2% CMA and transferred to Petri dishes of 9.0 cm in diameter containing 20 ml of 2% potato dextrose agar (2% PDA), maintained at 26°C in the dark and during 10 days. After the growth of the isolate, novel culture dishes of 4 mm in diameter were transferred to Petri dishes of 9.0 cm in diameter containing 20 ml of 2% water-agar (2% WA), maintained at 26°C in the dark and during 10 days (Araújo et al., 2008).

Protease production of *P. chlamydosporia* (VC4)

P. chlamydosporia (VC4) was cultured in flasks vials with 50 ml of liquid medium (0.3 g/L NaCl, 0.3 g/L $MgSO_4.7H_2O$, 0.3 g/L K_2HPO_4, 0.2 g/L yeast extract) supplemented with 0.2% gelatin. Gelatin was filtered through Millipore filter (with 45 µm aperture) before being added aseptically in autoclaved medium. The samples containing the isolate were incubated in the dark at 28°C in a rotary shaker at 120 rpm. After five days, the supernatant was collected and filtered using Whatman filter paper No. 1 at 4°C according to Esteves et al. (2009).

Obtaining of *Ascaris suum* eggs

A. suum eggs were recovered from the dissection of adult specimens, obtained during the necropsy of a collared peccary (*P. tajacu*), who died under natural conditions. The identification of adult parasites followed the standards described by Soulsby (1982). Subsequently, the eggs were analyzed for their integrity under a light microscope in 10x objective according to Urquhart et al. (1998).

Experimental assays

Enzymatic assay

An *in vitro* assay was performed to confirm the protease activity of the fungus *P. chlamydosporia* (VC4), where the protease activity was measured as described by Soares et al. (2014) modified. The volumes of the solutions used in this method were: 100 µL of proteases, 400 µL of Tris-HCl 100 mM (pH 7.0) buffer and 500 µL of 1% casein pH 8.0. The reaction medium was incubated for 60 min and the reaction stopped by adding 1 ml of 10% trichloroacetic acid solution. After 10 min, the reaction medium was centrifuged at 10,000 x *g* for 5 min, the supernatant collected and the absorbance determined spectrophotometrically at 280 nm. A standard curve was constructed varying the concentrations of tyrosine (15 to 200 µg/mL). One protease unit was defined as the amount of enzyme required to liberate 1.0 µg of tyrosine per minute under the assay conditions used. The assay was performed in triplicate.

Ovicidal assay

Five hundred *A. suum* eggs were transferred into Petri dishes of 4.5 cm in diameter. Then there was added 5 mL of proteases from *P. chlamydosporia* (VC4) to each Petri dish in the treated group which was sealed with Rolapack film and incubated at 26°C, in the dark, for 14 days. The control group contained 500 *A. suum* eggs in 10 ml of denatured enzymes in Petri dishes, which were incubated under the same conditions. Six replicates were performed for each group. After 14 days, the number of eggs of *A. suum* present in each Petri dish from treated and control groups was calculated according to the method described by Soares et al. (2014) and Mukhtar and Pervaz (2003). The eggs were counted by means of light microscopy. Data obtained in the experimental test were subjected to analysis of variance at significance levels of 1 and 5% probability and to non-parametric Friedman test with 1% probability (Ayres et al., 2003). The average reduction percentage of *A. summ* eggs was calculated according to the following equation:

$$\%\text{Reduction} = \frac{\text{Average of eggs from control} - \text{Average of eggs from treatment})}{\text{Average of eggs from control}} \times 10$$

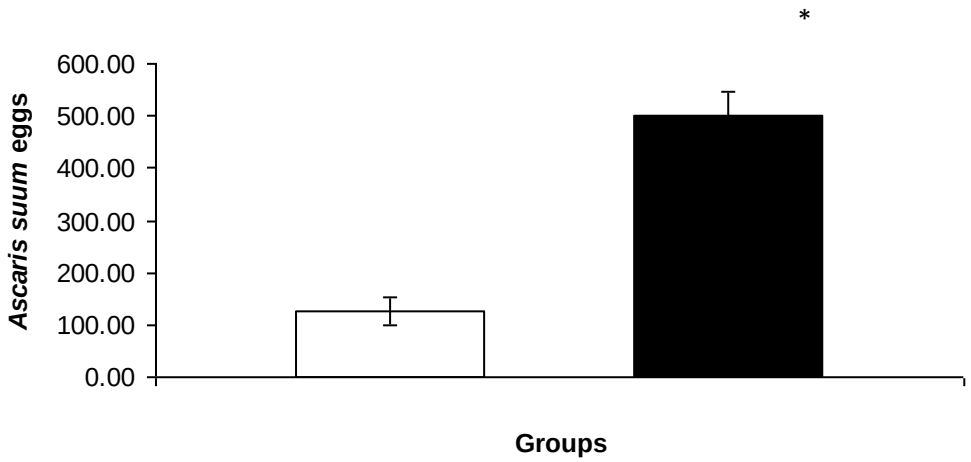

Figure 1. Ovicidal activity of *Pochonia chlamydosporia* (VC4) proteases on eggs of *Ascaris suum* in Petri dishes in treated group after 14 days of interaction and control group. Significant difference ($p < 0.01$) between the group treated with fungus and control (asterisk, Friedman test at 1% probability).

RESULTS

The proteases of *P. chlamydosporia* (VC4) showed ovicidal activity (destruction of eggs) in Petri plates from treated group after 14 days of interaction (Figure 1). However, destroyed eggs were not observed in the plates of the control group after the same time interval. On the other hand, the reduction percentage of 75% of the *A. suum* eggs in the treated group was observed compared to the control group. Additionally, the protease activity of the fungus *P. chlamydosporia* (VC4) was confirmed and measured in the *in vitro* assay. The value of the proteolytic activity was 9.38 U/mL, with a standard deviation of 0.47 U/mL.

Significant difference ($p < 0.01$) was found between the number of destroyed eggs (ovicidal activity) on the plates of the treated group compared to the control group. Furthermore, regarding the percentage of non-embryonated eggs in plates from treated group, the proteases of *P. chlamydosporia* (VC4) were effective in relation to the control group. The percentage of non-embryonated eggs were 78.7 and 83.5% for the treated and control groups, respectively.

DISCUSSION

In the present study, we demonstrated the enzymatic activity of proteases of *P. chlamydosporia* (VC4) in the destruction of *A. suum* eggs at the end of 14 days of interaction. This result is in agreement with those of Esteves et al. (2009), who worked with *P. chlamydosporia* grown in liquid medium supplemented with 0.2% gelatin and then demonstrated its action on hatching of *Meloidogyne* spp. eggs, a phytonematode. In addition, the proteolytic activity of the fungus P. chlamydosporia (VC4) was measured in order to confirm its enzymatic action. An activity value of 9.38 U/mL was observed; this value being similar to that found by Braga et al. (2012) using the same fungus. Nevertheless, in relation to the ovicidal activity of *P. chlamydosporia* on eggs from *Ascaris* genus, some studies have been conducted with this fungus in experimental assays in 2% solid water-agar (2% WA). Braga et al. (2007) showed that *P. chlamydosporia* (VC4) was effective in the destruction of *A. lumbricoides* eggs under laboratorial conditions, noting at the end of the experiment, a percentage of 26% in the destruction of eggs. In another study, Araújo et al. (2008) demonstrated the efficacy of the same fungus in the destruction of *A. suum* eggs in three day intervals (7, 14 and 21 days), and that at the end of 14 days, they reported 17.7% percentage of eggs destroyed. Furthermore, these studies have not studied the action of the fungus on the embryonation of eggs. In this study, we proved the destruction of the *A. summ* eggs by proteases of *P. chlamydosporia* (VC4) and its effectiveness in embryonation thereof, with 75% reduction percentage.

Braga et al. (2009b) reported that *P. chlamydosporia* (VC4) had proven efficacy in destroying *Austroxyuris finlaysoni* eggs, one oxyuridae of marsupials, in 2% WA. In that work, a percentage of 21.0% of eggs destroyed was registered after 15 days. This information is interesting because in that paper the authors discuss the difficulty of controlling worms in wild animals. However, the authors of this study mention by means of the results obtained a new verminosis control alternative in wild animals kept in zoos and in commercial breeding.

In several countries, the *Ascaris* genus has been mentioned in wild swine bred in captivity, causing abdominal

cramps and intestinal obstruction in these animals (Carlos et al., 2008). Furthermore, Mundim et al. (2004), reported that roundworms are very frequent and their eggs have long period of resistance in the environment, facilitating the infection of the animals and contributing to its high frequency. In this context, by means of the results obtained in this study, the employability of *P. chlamydosporia* and its proteases as an alternative of environmental control of *A. suum* eggs from wild swine bred in captivity (zoos and commercial breeding) is suggested. Moreover, this is the first report of the action of these enzymes from *P. chlamydosporia* on *A. suum* eggs of a wild swine (*P. tajacu*). However, further studies on these proteases will be the focus of other works.

Conflict of interests

The authors did not declare any conflict of interest.

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