

This Provisional PDF corresponds to the article as it appeared upon acceptance. Fully formatted PDF and full text (HTML) versions will be made available soon.

Concomitant production of lipase, protease and enterocin by *Enterococcus faecium* NCIM5363 and *Enterococcus durans* NCIM5427 isolated from fish processing waste

International Aquatic Research 2012, **4**:14 doi:10.1186/2008-6970-4-14

Vrinda Ramakrishnan (vrinda.r2@gmail.com)
Bijinu Balakrishnan (bijinu.b@gmail.com)
Amit Kumar Rai (amitrkvs@gmail.com)
Bhaskar Narayan (bhaskar@cftri.res.in)
Prakash M Halami (kash13@yahoo.com)

ISSN 2008-6970

Article type Original research

Submission date 30 April 2012

Acceptance date 7 August 2012

Publication date 28 August 2012

Article URL <http://www.intaquares.com/content/4/1/14>

This peer-reviewed article can be downloaded, printed and distributed freely for any purposes (see copyright notice below).

For information about publishing your research in *International Aquatic Research* go to

<http://www.intaquares.com/authors/instructions/>

For information about other SpringerOpen publications go to

<http://www.springeropen.com>

Concomitant production of lipase, protease and enterocin by *Enterococcus faecium* NCIM5363 and *Enterococcus durans* NCIM5427 isolated from fish processing waste

Vrinda Ramakrishnan¹
Email: vrinda.r2@gmail.com

Bijinu Balakrishnan²
Email: bijinu.b@gmail.com

Amit Kumar Rai²
Email: amitrkvs@gmail.com

Bhaskar Narayan^{2*}
* Corresponding author
Email: bhaskar@cftri.res.in

Prakash M Halami^{1*}
* Corresponding author
Email: kash13@yahoo.com

¹ Department of Food Microbiology, Central Food Technological Research Institute (Council of Scientific and Industrial Research), Mysore, Karnataka 570 020, India

² Department of Meat, Fish and Poultry Technology, Central Food Technological Research Institute (Council of Scientific and Industrial Research), Mysore, Karnataka 570 020, India

Abstract

Enterococci are widely distributed in the environment ranging from foods to humans and are gaining industrial importance due to their technological traits. In the present study, enterococci (*Enterococcus faecium* NCIM5363 (EF-63) and *Enterococcus durans* NCIM5427 (ED-27)) which are native to fish processing waste with an ability to produce lipase, protease and enterocin concomitantly were characterised. Lipase assay was performed by titrimetry and protease assay and was estimated using haemoglobin and casein as substrates in the presence of buffers at acidic, basic and neutral pH. Furthermore, enterocin produced by the isolates was characterised. Enterocin was also checked for its stability at different pH, temperature and proteolytic enzymes. Lipase production was found to be 22 and 10 U/ml in the absence of tributyrin and increased to 40 and 24 U/ml in its presence for EF-63 and ED-27, respectively, indicating that the lipase produced is substrate dependent. Protease production was confirmed by protease assay, and the protease produced showed more affinity towards the acidic substrate. Enterocin produced was stable at low pH (2 to 3) and high temperature (121°C, 15 min) and had a molecular weight of approximately 6 kDa. It exhibited antibacterial activity against both Gram-positive and Gram-negative food-borne

pathogens. Proteinase K inactivated enterocin completely, whereas trypsin did not. Novelty of this work lies in the immense industrial importance these cultures hold as they are capable of producing lipase, protease and enterocin apart from being useful in recovering proteins and lipids from fish processing wastes.

Keywords

fish viscera, LAB, enterocin, protease, lipase, antibacterial, antioxidative.

Background

Enterococci are a diverse group of lactic acid bacteria (LAB) that possess numerous technological traits apart from contributing to the organoleptic properties of fermented food products. Their importance as either starter or adjunct cultures in the dairy industry is highly important, as they can produce several enzymes that can interact with milk components promoting important biochemical transformations like acidification, proteolytic/peptidolytic activity, lipolytic/esterolytic activity, citrate/pyruvate metabolism and bacteriocin production (Giraffa 2003). Among the 28 different reported species of enterococci, *Enterococcus faecalis*, *Enterococcus faecium* and *Enterococcus durans* are found to possess maximum proteolytic and lipolytic activity (Moreno et al. 2006). However, very few reports exist on the simultaneous production of protease and lipase along with bacteriocin by LAB species.

Lipases in enterococci are often strain dependent (Carrasco de Mendoza et al. 1992). Most of them show activity against tributyrin to tristearin in a decreasing order. Enterococci are capable of producing both lipases and esterases; esterases have a direct effect on flavour development and rheology, whereas lipases influence molecular organisation thereby having an effect on the texture of the final product. Enterococci possess greater lipolytic and proteolytic activities as compared to other LAB (Tsakalidou et al. 1994). Lipase-producing enterococci are of utmost importance not only in the food industry, but also in oleochemical, leather, detergent, perfume, cosmetic and organic industries. They are also useful in environmental management, biomedical applications and biosensors (Talon et al. 1996). Proteases of LAB help in casein degradation, along with secondary degradation of amino acids, have a major role in imparting flavour to various dairy products. Enterococci are found to have lesser proteolytic activity in comparison with other LAB such as *Lactobacillus* and *Lactococcus*. In general, LAB that produce proteases are mainly used in the food industry as starter cultures in cheese manufacturing (Wilkinson et al. 1994).

Numerous enterococcal strains found in a variety of fermented and non-fermented foods have attracted increased research interest for their use in food preservation as they produce more than one bacteriocin per strain (Cleveland Monteville et al. 2001). Bacteriocin produced by enterococci, commonly referred to as enterocins, is gaining interest because of its industrial potential. Also, these strains have been used as co-cultures in food fermentation in order to reduce the risk of *Listeria* (Leroy et al. 2003). Thus, application of enterocins, either as food additive or through *in situ* production by an appropriate LAB isolate during food fermentations, appears promising (Callewaert et al. 2000).

Apart from the above, lipolytic and proteolytic enterococci can also be used in efficient waste management as other technologies recommended require a high initial investment (Arruda et al. 2007). Transformation of these wastes into products to be incorporated as ingredient in

animal feeds is a method of minimising the disposal related issues (Ristic et al. 2002). One of the most feasible methods that uses LAB not only utilises and breaks down the waste effectively, but also helps in the recovery of bioactive molecules that include protein, lipids, collagen and bacteriocin-like substances (Amit et al. 2010). LAB are preferred over other bacteria as they are Gram-positive obligate or facultative anaerobes dominant in the gastrointestinal microbiota of both man and terrestrial farm animals. Also, they easily adapt to the intestinal environment of both aquatic and domestic animals, making them favourable for use in probiotic aquaculture and poultry feeds. Moreover, isolation of enterococci from fish waste source increases their chances of adaptability and greater utilisation of these wastes to enable *in situ* enrichment and recovery of bioactive molecules.

In the present study, two different strains of enterococci that concomitantly produce lipase, protease and enterocin were identified, and their metabolites that have potential for applications in either food or pharmaceutical products were also characterised.

Methods

Sample collection, chemicals and reagents

Visceral wastes from freshwater Indian major carps (Catla, *Catla catla*; Mrigal, *Cirrhinus mrigala*; Rohu, *Labeo rohita*) were procured from local markets and transported in sterile pouches under iced conditions. The visceral wastes were homogenised using a pre-sterilised warring blender (Stephen Mill, UM5 Universal, Hong Kong) for further use. All microbiological media were from Hi-Media (M/s Hi-Media, Mumbai, India). Polymerase chain reaction (PCR) reagents were from M/s Bangalore Genei, Bangalore (India). Sodium dodecyl sulphate (SDS), agarose and acrylamide were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Ethylenediaminetetraacetic acid (EDTA) and hydrogen peroxide were purchased from Merck (Mumbai, India). The pathogens used for the tests were obtained from various collection centres and maintained as BHI glycerol stocks at -20°C and subcultured periodically. All other chemicals, solvents and reagents used in the study were of analytical grade, unless otherwise mentioned.

Bacterial strains and culture conditions

The lipolytic, proteolytic and bacteriocin-producing LAB strains used in the study were isolated from the homogenised freshwater fish viscera. The homogenised samples were serially diluted and pour plated onto two different media, i.e. de Man-Rogosa-Sharpe (MRS) agar in combination with tributyrin (1%) as well as MRS in combination with skimmed milk (1%), for the detection of lipolytic and proteolytic LAB, respectively. LAB strains with potent proteolytic and lipolytic activity were selected based on the diameter of zone of clearance on the media. The proteolytic isolates were then streaked tributyrin-based media and lipolytic isolates onto skimmed milk media for the selection of isolates possessing both the properties. These isolates were then checked for their antibacterial activity. The enterocin-producing *E. faecium* (Microbial Type Culture Collection Center (MTCC5153)) was used as a positive control in assays aimed at detecting bacteriocin production. Two isolates namely EF-63 and ED-27 having proteolytic, lipolytic and bacteriocin-producing ability were selected. The LAB cultures were maintained in 60% (v/v) glycerol stocks at -20°C and sub-cultured periodically.

Biochemical and molecular characterization of *Enterococcus* strains

The selected cultures were subjected to various standard biochemical and physiological methods as per the schemes outlined in the *Bergey's Manual of Systematic Bacteriology* (Holt and Bergey 1994). The isolates were subjected to Gram's staining and catalase test and were tested for their ability to ferment a variety of carbohydrates and for their capability of growth at 10°C and 45°C in media containing 6.5% NaCl at pH 9.6. After this initial characterisation, the strains were further characterised using molecular methods.

Standard methodologies were followed for molecular techniques (Sambrook and Russell 2001). Total genomic DNA from the enterococcal strains was isolated as described by Mora et al. (1998). For amplification of the 16S rRNA gene, PCR was performed using the primers as well as amplification conditions as previously described (Amit et al. 2009). The PCR amplicons were analysed on 1.5% agarose gel by electrophoresis. The PCR product was eluted using the Qiagen gel extraction kit (M/s Qiagen, Hilden, Germany) and sequenced at the Vimta Labs, Hyderabad (India).

Activity, PCR analysis and molecular weight estimation of lipase

Lipase activity in culture filtrate was assayed based on the measurement of free fatty acid release due to enzymatic hydrolysis of triglycerides in stabilised emulsion of vegetable oil. Emulsion preparation and assay were carried out as per the method described by Yamada et al. (Shimizu et al. 1979). A unit of lipase activity was defined as the volume of 0.01 N NaOH required to neutralise the free fatty acids formed on hydrolysis of oil per milligram of protein in extract. Lipase assays were conducted for both the cultures at different time intervals. The cultures were incubated for 72 h at 30°C and 40°C with pH that range from 6 to 9. The samples were drawn every 24 h of interval, and lipase assay was performed.

For the amplification of the lipase gene, primers used were as described by Shi et al. (2010). However, the PCR was performed with slight modifications in the conditions, with an initial denaturation at 95°C for 5 min; this was followed by 35 cycles of 94°C for 5 min, 60°C for 30 s and 72°C for 1 min, and a final extension of 72°C of 5 min occurred. The PCR product was analysed on 1% agarose gel.

To estimate the molecular weight of lipase extracted from the cultures, SDS-PAGE was performed, followed by activity assay in which the gel was overlaid with 2% tributyrin and incubated for 48 h.

Protease activity

The assays for acidic, neutral and alkaline proteases were carried out as described by (Bhaskar et al. 2007). Casein was used as a substrate for neutral and alkaline proteases, and haemoglobin for acidic proteases and acetate (pH 4.5) and phosphate (pH 6.5 and 8) buffers was used for the assay. The absorbance was converted to microgram tyrosine using a standard curve. One unit (U) of proteolytic activity was defined as microgram tyrosine liberated per minute per millilitre of the enzyme extract. Specific activity was expressed as units per mg protein (U mg^{-1} protein) of the culture filtrate. Protease assay was done at different time intervals and pH ranges.

Antagonistic effect of enterocin and its mode of action

Preparation of samples and agar well assay

Cell-free supernatants (CFS) were collected, and bacteriocin activity was tested using the agar well diffusion method (Geis et al. 1983) against *Listeria monocytogenes* Scott A and several pathogens listed in Table 1. Crude bacteriocin was extracted either by chloroform extraction (Burianek and Yousef 2000) or concentrated by ammonium sulphate precipitation (70%) (Muriana and Klaenhammer 1991). Bacteriocin activity was measured in arbitrary units per millilitre (AU ml⁻¹) and is defined as the reciprocal of the highest dilution exhibiting complete inhibition of the indicator lawn. Bacteriocin concentration was estimated colorimetrically by Lowry et al. (1951).

Table 1 Antibacterial spectrum of bacteriocin produced by EF-63 and ED-27 (n = 3)

Bacterial pathogens	Zone of inhibition (mm)	
	EF-63	ED-27
<i>L. monocytogenes</i> Scott A ^a	20	20
<i>Listeria innocua</i> FB21 ^b	20	19
<i>Listeria murrayi</i> FB69 ^b	20	20
<i>Escherichia coli</i> MTCC 118 ^c	16	ND
<i>Aeromonas hydrophilia</i> B445 ^d	18	17
<i>Staphylococcus aureus</i> FRI 231 ^b	14	15
<i>Salmonella paratyphi</i> FB 254 ^b	9	10
<i>Pediococcus acidilactici</i> PAC 1.0 ^e	14	16
<i>Yersinia enterocolitica</i>	9	7

^aStrain obtained courtesy of Dr. AK Bhunia, Purdue University, IN, USA; ^bFood Microbiology Department, Culture Collection CFTRI, Mysore; ^cMTCC, India; ^dNRRL, Northern Regional Research Laboratory; ^eStrain obtained courtesy of D Mora, University of Milano, Milan, Italy. ND, not detected.

Effect of temperature, pH and proteolytic enzymes on partially purified enterocin

The partially purified enterocin was subjected to the action of temperature, pH and proteolytic enzymes. The stability of enterocin was tested under various ranges of temperature and pH, and time intervals as indicated in Table 2. Similarly, sensitivity of enterocin against various proteolytic enzymes such as trypsin, pepsin and proteinase K was also performed. The samples were then assayed for activity by agar well assay (Geis et al. 1983) with appropriate controls using *L. monocytogenes* Scott A as the test organism. Untreated enterocin served as the control, and all experiments were carried out in triplicates.

Table 2 Effect of different parameters on antibacterial activity of enterocin of EF-63 against *L. monocytogenes* Scott A

Effect of proteases ^a		Effect of pH ^b		Effect of temperature ^b	
Enzyme	Zone(mm)	pH	Activity	Temperature (°C)	Activity
Control	20	Control	3,200	Control	3,200
Trypsin	0	2.0	ND		
Pepsin	0	3.0	1,600	60, 15 min	3,200

Proteinase K	0	4.0	3,200		
		5.0	3,200	60, 30 min	3,200
		6.0	3,200		
		7.0	3,200	100, 15 min	3,200
		8.0	3,200		
		9.0	3,200	100, 15 min	3,200
		10.0	3,200	121, 20 min	3,200

^aAll the proteolytic activity estimations were done at 37°C and incubation time of 4 h; ^bantibacterial activity is expressed in arbitrary units (AU/ml). ND, not detected. In the case of enzymes, bacteriocin (10 µl; protein content, 10 µg) was treated with 10 µl of 10% enzyme.

Tricine-SDS-PAGE

Tricine-SDS-PAGE was performed, and activity assay was done against *L. monocytogenes* Scott A and *E. coli* MTCC 118 as indicator organisms, as described previously (Schagger and von Jagow 1987). Similarly, molecular weight of the bacteriocin produced by the two potent isolates was also determined by SDS-PAGE by loading the sample in duplicates along with lower range protein molecular weight markers (M/s GeNei, Bangalore, India).

Sensitivity to antimicrobial agents

The sensitivity to different antibiotics (amikacin (10 µg), amoxicillin (10 µg), bacitracin (10 U), cephalothin (30 µg), erythromycin (15 µg), novobiocin (30 µg) and vancomycin (30 µg) (M/s HiMedia, Mumbai, India) was done as described earlier (Amit et al. 2009). Briefly, MRS agar plates containing 15 ml of MRS agar were overlaid with 8 ml of soft agar (0.8%) containing 50 µl of freshly grown culture, and the plates were incubated at 4°C for the soft agar media to set. Antibiotic discs were placed on solid media and incubated under microaerophilic conditions in an anaerobic jar (Hi-Jar; M/s Hi-Media, Mumbai) at 37°C for 24 h. Inhibition zones were measured, and results were interpreted as per manufacturer's protocol as resistant (R), sensitive (S) or intermediate sensitive (IS) based on the zone of inhibition.

In vitro antioxidant properties

Partially purified enterocin was used for assaying DPPH scavenging activity and total antioxidant property. The scavenging effect (percentage) was calculated as per the formulae in Duan et al. (2006). Total antioxidant activity (TOA) was performed as per the method described by Amit et al. (2009). TOA was expressed as milligram of ascorbic acid equivalents per milligram of protein in sample.

Bacterial culture deposition and accession numbers

The nucleotide sequences were analysed by the basic local alignment search tool (BLAST) on the National Center for Biotechnology Information (NCBI) database (Altschul et al. 1997). The results of BLAST analysis based on 16S rRNA gene sequences stand deposited in the GenBank database (1982) maintained by NCBI, Bethesda, MD, USA with accession numbers HQ005360 and JN100142. The two enterococcal strains are deposited in the National Collection of Industrial Microorganisms at NCL, Pune, India for public access under the accession numbers *E. faecium* NCIM5363 and *E. durans* NCIM5427.

Results and discussion

Identification of the lipolytic, proteolytic and bacteriocinogenic LAB strains

A total of 119 LAB was isolated from freshwater fish visceral wastes of which 33 enterococci were selected based on their strong proteolytic, lipolytic and antibacterial properties. All the isolates were Gram-positive, coccoidal, catalase-negative and oxidase-positive and able to utilise 0.04% sodium azide. Their growth at 50°C, 8% NaCl and pH of 9.6 indicated their ability to tolerate relatively higher temperature and salt concentrations, a characteristic feature of enterococci. Casein and skimmed milk hydrolysis indicated that they were potent producers of proteolytic enzymes. Among the 33 different enterococcal species, EF-63 and ED-27 showed higher proteolytic and lipolytic properties apart from being antagonistic towards Gram-positive and Gram-negative food-borne pathogens as well as other LAB and hence were considered for further studies. Apart from biochemical characterization molecular methods such as 16S rRNA approach clearly indicated that the strains belonged to *E. faecium* and *E. durans*. Enterococci are a group of LAB that is gaining importance in the food industry (mainly cheese production) due to its technological traits. The results of our study hold significant in the fact that the cultures have proteolytic, lipolytic and antibacterial properties which can be effectively made use of in the food industry as starter cultures for flavouring, texture, rheology and shelf life extension of cheeses (Moreno et al. 2006). Moreover, bacteriocin or bacteriocin-like products of Gram-positive bacteria, especially those that have broad antibacterial spectrum, are an active area of applied microbiological research. The potential for either the discovery or genetic engineering of novel peptides with commercially desirable antibacterial activities offers an irresistible lure.

Lipolytic and proteolytic properties of isolates

Lipase assay indicated that EF-63 showed more lipolytic activity than ED-27. Lipase activity was carried out in the presence and absence of a substrate (1%) which showed that lipase production by the isolates was substrate dependent (Figure 1A). At pH 6 and 9, maximal lipase production was observed. At the end of 72 h at 30°C, lipase activity was found to be 10 U/ml in the case of ED-27 and 22 U/ml in the case of ED-27 at pH6 in the absence of a substrate and 24 U/ml and 40 U/ml in the presence of tributyrin, respectively. At 40°C, lipase activity was found to be maximal after 24 h of 8 and 12 U/ml in the case of ED-27 and Ef-63, respectively. Hence, 30°C was found to be the optimum temperature for lipase production.

Figure 1 Lipolytic activity, electrophoretic profile and PCR analysis. (A) Lipolytic activity of EF-63 and ED-27 isolated from fresh water fish viscera ($n = 3$). (B) Electrophoretic profile of lipase produced by isolates and its activity assay. (C) PCR analysis indicating gene obtained from the isolate coding for lipase.

PCR showed an amplification of 700 bp (Figure 1C) as expected with a putative molecular weight of approximately 22.86 kDa as determined by SDS-PAGE (Figure 1B). This was indicative of the presence of the lipase gene and lipase production.

Very few studies on lactic acid bacterial lipases have been carried out. Studies by Tsakalidou et al. (1994) have concluded that even though LAB are weakly lipolytic, the enterococcal strains showed a significantly higher activity than the strains of most other genera of LAB. Similarly, studies concerning the proteolysis of LAB are mainly restricted to *Lactococcus* and

Lactobacillus (Moreno et al. 2006). Microbial lipases are used in the dairy industry extensively for the hydrolysis of milk fat, and current applications include acceleration of cheese ripening and lipolysis of butter, fat and cream (Aravindan et al. 2007). Apart from the far-reaching benefits provided by the cultures under study, the source from which the cultures are isolated also evokes a great interest. The basic idea of our work was to effectively utilise wastes from the fish industry and convert them into value-added products. Since the two strains isolated are highly lipolytic and proteolytic, they are capable of *in situ* enrichment of the lipids and thereby PUFA enrichment. Hence, we aim at the complete disposal and effective management as well as utilisation of fish industry wastes using LAB as starter culture.

Protease producing abilities of cultures at different pH (6 to 9) and temperature (30°C and 40°C) were studied, and the results indicated that both Ef-63 and ED-27 produce a protease that is active between pH 6 and 9. However, the activity at pH 6 was significantly ($p < 0.05$) higher compared with that of other pH ranges. Also, the protease showed higher activity with haemoglobin as the substrate as compared with casein as the substrate (data not shown), presumably indicating that the protease produced is more of the acidic nature. Hence, protease activity using haemoglobin as substrate was carried out. Maximal protease production was observed between 24 and 48 h in the case of EF-63, whereas it was observed at 24, 48 and 72 h with the optimum at 48 h in the case of ED-27 (Figure 2A). At 30°C, maximal protease production was observed at 48 h in the case of both cultures ($p < 0.05$). At 40°C, EF-63 showed maximal protease activity from 24 to 48 h which later reduced at 72 h, whereas ED-27 showed maximal protease activity at 40°C in 24 h and at 30°C in 48 h (Figure 2B).

Figure 2 Specific activity of protease (units per milligram of protein). It is produced by (A) EF-63 and (B) ED-27 as affected by different pH (6, 7, 8 and 9) and temperature (30°C and 40°C) ranges.

LAB proteases are employed to hydrolyse whey protein in the dairy industry as hydrolysed whey - which is used to prepare supplements for infant formulas and medical uses - which is less likely to cause allergic reactions. Moreover, the applicability of enterococci as starter or adjunct cultures has increased as they both have proteolytic and esterolytic properties. Also, the strains tested positively affected taste, aroma, colour and structure of fully ripened cheeses as well as the overall sensory profile in comparison with the control (Moreno et al. 2006). The cultures under study have great significance as they are capable of producing proteases which are stable at acidic pH. As per previous reports, *E. faecium* was found to possess more lipolytic activity than *E. durans*. On the other hand, *E. durans* showed more proteolytic properties than *E. faecium* (Sarantinopoulos et al. 2001); however, the cultures possessing both the properties were not reported. To our knowledge, this the first report stating lipolytic, proteolytic and antibacterial activities in the same strain of *Enterococcus*, and there have been no reports of concomitant production of protease and lipase by enterococci.

Enterocin production and its antagonistic properties

The two strains were tested for their antimicrobial activity against the pathogens listed in Table 1. Except in the case of *Y. enterocolitica* and *Salmonella typhi*, the two strains exhibited strong zones of inhibition against the growth of other pathogens. EF-63 was active against *E. coli* MTCC118, whereas ED-27 did not cause any inhibition. The antibacterial

activity of the cultures was found to be in its maximum in the case of *L. monocytogenes* Scott A. Since only the enterocin from EF-63 was found to be active against *E. coli*, it was characterised as indicated in Table 2. Enterocin was found to retain its activity even after heat treatment, indicating that it is thermostable. It could also withstand the variations in pH without losing its activity. The stability over a broad range of pH has a strong influence on bacteriocin activity, and bacteriocins such as nisin, sakacin P and curvacin A showed highest activity at an acidic pH even against *E. coli* and *Salmonella enterica* (Ananou et al. 2005). It was unaffected by trypsin, whereas proteinase K showed complete inhibition even at 2 h. Pepsin showed reduction in activity after 2 h and complete loss of activity after 4 h as indicated in Table 2.

Tricine gel analysis indicated the molecular weight of enterocin to be less than 6.5 kDa (Figure 3). The zone of inhibition also corresponded with the value of 6.5 kDa when the gel was overlaid with the indicator organism *L. monocytogenes* Scott A (lane A), and the same position of zone also showed activity against *E. coli* MTCC 118 (lane B).

Figure 3 Tricine-SDS-PAGE of enterocin from EF-63. *L. monocytogenes* Scott A (lane A) and *E. coli* MTCC118 (lane B) were used as indicators.

Previous reports have stated that the outer membrane of *E. coli* was rigid and the enterocin could kill the *E. coli* only when used in combination with EDTA, sodium tripolyphosphate, low pH, high pH and heat (Ananou et al. 2005). The main novelty behind this work is that the bacteriocin produced by these strains is active against *E. coli* as very few reports of bacteriocins produced by Gram-positive bacteria active against Gram-negative bacteria have been cited (Minahk et al. 2000). Moreover, the cultures also showed activity against *Staphylococcus* which indicates their potential application as probiotics in raw or processed foods destined for human and animal consumption. Therefore, the application of enterocin-producing strains for the preservation in meat fermentation can be beneficial to prevent growth of spoilage and pathogenic microorganisms such as *S. aureus* by natural means.

Currently, fish are protected from bacterial diseases either through vaccination or with chemotherapeutic treatment; neither of the treatments is completely effective. Vaccinations are ineffective when applied to immunologically immature fish, whereas chemotherapeutic agents such as antibiotics lead to the evolution of resistance among pathogenic bacteria (Angulo 2000; Balcazar et al. 2006). Hence, the requirement for a probiotic feed is on high demand, making it all the more necessary for the isolation and characterization of a lipolytic, proteolytic and antibacterial LAB (Balcazar et al. 2008) from fish processing wastes and its application as aqua- and poultry feeds. High antibacterial ability of the cultures against the major fish pathogen *A. hydrophila* indicates that the enterocins produced by these cultures could be incorporated into aquafeeds. Fish meal is the most abundant animal protein source for the manufacturing of rations for domestic animals.

Antibiotic sensitivity and antioxidant properties

Antibiotic sensitivity assay indicated that the native isolates were resistant to amikacin. Further, all were sensitive to amoxicillin, novobiocin and vancomycin (data not shown). All isolates were intermediately resistant to erythromycin. The sensitiveness of all four isolates to vancomycin is indicative of their suitability for use as a probiotic in animal/fish feeds. One of the common threats behind the use of enterococci is due to the presence of vancomycin resistance, and it is necessary to evaluate the target cultures for vancomycin resistance (Franz

et al. 2003). The strains reported in our study also showed vancomycin sensitivity, a very important demarcating feature for selection as a probiotic. Since the two isolates were sensitive to vancomycin, it suggests their suitability for use as a probiotic in animal/fish feeds. Similarly, their resistance to several other antibiotics indicates their potential for use as probiotic culture in livestock feeds for animal health management. They were also evaluated for their haemolytic activity and did not show hydrolysis of sheep blood *in vitro*, this being another desirable phenotype in terms of lack of infectivity and pathogenicity.

DPPH radical scavenging activity of the enterocin was assessed. Both the cultures showed good antioxidant properties. The DPPH scavenging of the isolates was in the range of 90.6% to 91.8% as compared to $95.4 \pm 1.2\%$ scavenging exhibited by α -tocopherol at 200 μg . In the case of total antioxidant property, *E. faecium* NCIM5363 showed more activity than *E. durans* (Figure 4A). Even the total antioxidant activity followed the same trend where activity of *E. faecium* NCIM5363 was high (Figure 4B).

Figure 4 *In vitro* antioxidant properties of culture filtrates of EF-63 and ED-27. (A) Total antioxidant property (as milligrams of ascorbic acid equivalents per milligram of protein), (B) DPPH scavenging (percentage) activity.

Fish silage can be used as an alternative protein source in the formulation of rations for monogastrics. However, due to lipid oxidation that can compromise the nutritional value of the ration, removal of the lipid fraction is required. This additional step can be avoided since the cultures were also found to possess good antioxidant properties, thereby protecting the lipid layer from auto-oxidation. Lipid oxidation can provoke the formation of peroxides, which form physical and covalent bonds from proteins. Covalent bonds between oxidised products and proteins can destroy amino acids, such as tryptophan, oxidise methionine and bind lysine to other compounds, making these amino acids unavailable. Hence, through the usage of these cultures, there is no requirement for the additional inclusion of antioxidants in the preparation of fish silage.

Conclusions

The present study reveals the potential for complete and proper utilisation of fish processing wastes through the use of *E. faecium* as starter culture. The by-products that are formed as a result of degradation of fish wastes find immense applications in the fields of biotechnology. The bacteriocins formed by them can be effectively used in food preservation as they are active against both Gram-positive and Gram-negative bacteria. Although lipases have several interesting potential applications in the food, their industrial uses still remain limited by their high production costs, commercialization in small amounts and low performance of some lipase-mediated processes. Thus, the direct application of these highly lipolytic cultures on fish waste substrate helps in the rapid production of lipases, and downstream processing also becomes simpler and cost-effective. Hence, these lipase, protease and enterocin-producing cultures have immense applications in food, detergent, pharmaceutical, leather, textile, cosmetic and paper industries, making this study highly significant.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

VR, AKR and BB carried out the bench work. BN and PMH have significant contributions in conceptualising, designing experiments and writing the paper. All authors read and approved the final manuscript.

Acknowledgements

The authors thank the Department of Biotechnology (DBT), Government of India for partially funding this work through grant no. BT/PR 9474/AAQ/03/345/2007 to the corresponding author. Vrinda Ramakrishnan acknowledges the University Grants Commission (UGC) for the CSIR-UGC fellowship. The authors thank the director of CFTRI for the encouragement and permission to publish the work.

References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nuc Acids Res* 25:3389–3402

Amit KR, Bhaskar N, Halami PM, Indrani K, Suresh PV, Mahendrakar NS (2009) Characterization and application of a native lactic acid bacterium isolated from tannery fleshings for fermentative bioconversion of tannery fleshings. *Appl Microbiol Biotechnol* 83:757–766

Amit KR, Swapna HC, Bhaskar N, Halami PM, Sachindra NM (2010) Effect of fermentation ensilaging on recovery of oil from fresh water fish viscera. *Enz Microbial Technol* 46:9–13

Ananou S, Galvez A, Martinez-Bueno M, Maqueda M, Valdivia E (2005) Synergistic effect of enterocin AS-48 in combination with outer membrane permeabilizing treatments against *Escherichia coli* O157:H7. *J Appl Microbiol* 99:1364–1372

Angulo F (2000) Antimicrobial agents in aquaculture: potential impact on public health. *APUA Newslett* 18:1–4

Aravindan R, Anbumathi P, Viruthagiri T (2007) Lipase applications in food industry. *Ind J Biotech* 6:141–158

Arruda LF, Borghesi R, Oetterer M (2007) Use of fish waste as silage - a review. *Brazilian Archives Biol Technol* 50:879–886

Balcazar JL, de Blas I, Ruiz-Zarzuola I, Cunningham D, Vendrell D, Muzquiz JL (2006) The role of probiotics in aquaculture, *Vet. Microbiol* 114:173–186

Balcazar JL, Vendrell D, de Blas I, Ruiz-Zarzuola I, Muzquiz JL, Girones O (2008) Characterization of probiotic properties of lactic acid bacteria isolated from intestinal microbiota of fish. *Aquaculture* 278:188–191

Bhaskar N, Sudeepa ES, Rashmi HN, Tamil Selvi A (2007) Partial purification and characterization of protease of *Bacillus proteolyticus* CFR3001 isolated from fish processing waste and its antibacterial activities. *Bio Res Technol* 98:2758–2764

Burianek LL, Yousef AE (2000) Solvent extraction of bacteriocins from liquid cultures. *Lett App Microbiol* 31:193–197

Callewaert R, Hugas M, De Vyust L (2000) Competitiveness and bacteriocin production of enterococci in the production of Spanish-style fermented sausages. *Inter J Food Microbiol* 7:33–42

Carrasco de Mendoza MS, Scarinci MS, Huerto-Garat HE, Simonetta AC (1992) Technological properties in enterococci in lactic starters acidifying and lipolytic activities. *Microbiologie Ailments Nutrition* 10:289–293

Cleveland Monteville TJ, Nes IF, Chikindas ML (2001) Bacteriocins: safe, natural antimicrobials for food preservation. *Int J Food Microbiol* 71:1–20

Duan XJ, Zhang WW, Li XM, Wang BG (2006) Evaluation of antioxidant property of extract and fractions obtained from a red alga, *Polysiphonia urceolata*. *Food Chem* 95:37–43

Franz CMAP, Stiles ME, Schleifer KH, Holzappel WH (2003) Enterococci in food—a conundrum for food safety. *Int J Food Microbiol* 88:105–122

Geis AJ, Singh R, Teuber MJ (1983) Potential of lactic streptococci to produce bacteriocins. *Appl Environ Microbiol* 45:205–211

GenBank database (1982). NCBI, Bethesda, <http://www.ncbi.nlm.nih.gov/Genbank>

Giraffa G (2003) Functionality of enterococci in dairy products. *Int J Food Microbiol* 88:215–222

Holt JG, Bergey DH (1994) *Bergey's manual of determinative bacteriology*, 9th edn. William and Wilkins, Baltimore

Leroy F, Moreno MRF, De Vyust L (2003) *Enterococcus faecium* RZS C5, an interesting bacteriocin producer to be used as a co-culture in food fermentation. *Intern J Food Microbiol* 8:235–240

Lowry OH, Fan AL, Randall RJ, Rosebrough NJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:256–275

Minahk CJ, Farias ME, Sesma F, Morero RD (2000) Effect of enterocin CRL35 on *Listeria monocytogenes* cell membrane. *FEMS Microbiol Lett* 192:79–83

Mora D, Parini C, Fortina MG, Manachini PL (1998) Discrimination among pediocin AcH/PA-1 producer strains by comparison of pedB and pedD amplified genes and multiplex PCR assay. *Syst Appl Microbiol* 21:454–460

Moreno FMR, Sarantinopoulos P, Tsakalidou E, De Vyust L (2006) The role and application of enterococci in food and health. *Int J Food Microbiol* 106:1–24

Muriana PM, Klaenhammer TP (1991) Purification and partial characterization of lactacin F, a bacteriocin produced by *Lactobacillus acidophilus* 11088. *Appl Environ Microbiol* 57:114–121

Ristic MD, Filipovic SS, Sakac MLJ (2002) Liquid protein feedstuffs from freshwater fish by-products as a component of animal feed. *Romanian Biotechnol Letts* 7:729–736

Sambrook J, Russell DW (2001) *Molecular cloning: a laboratory manual*, vol 1. Cold Spring Harbor Laboratory Press, New York

Sarantinopoulos P, Andrighetto C, Georgalaki MD, Rea MC, Lombardi A, Cogan TM, Kalantzopoulos G, Tsakalidou E (2001) Biochemical properties of enterococci relevant to their technological performance. *Int Dairy J* 11:621–647

Schagger H, von Jagow G (1987) Tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* 166:368–379

Shi B, Wu W, Wen J, Shi Q, Wu S (2010) Cloning and expression of a lipase gene from *Bacillus subtilis* FS1403 in *Escherichia coli*. *Ann Microbiol* 60:399–404

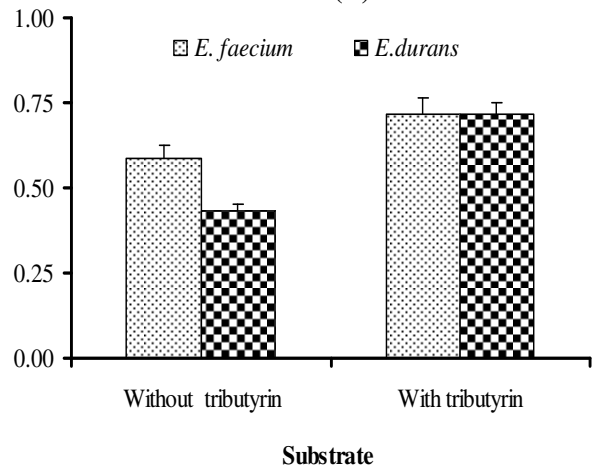
Shimizu S, Inoue K, Tani Y, Yamada H (1979) Enzymatic microdetermination of serum free fatty acids. *Analytic Biochem* 98:341–345

Talon R, Montel MC, Berdague JL (1996) Production of flavour esters by lipases of *Staphylococcus warneri* and *Staphylococcus xylosus*. *Enzyme Microb Technol* 19:620–622

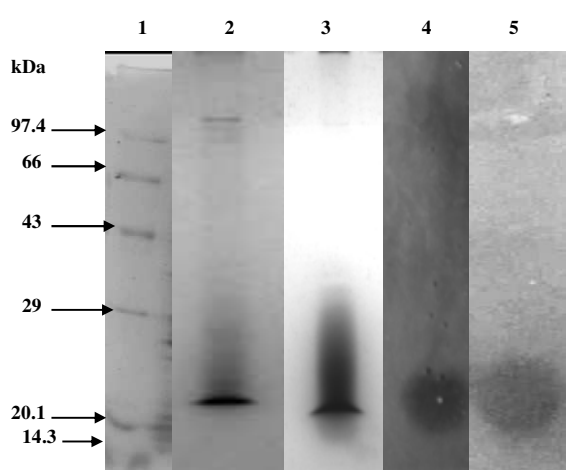
Tsakalidou E, Manolopoulou E, Kabaraki E, Zoidou E, Pot B, Kersters K, Kalantzopoulos G (1994) The combined use of whole cell protein extracts for the identification (SDS-PAGE) and enzyme activity screening of lactic acid bacteria isolated from traditional Greek dairy products. *Syst Appl Microbiol* 17:444–458

Wilkinson MG, Guinee TP, O'Callaghan DM, Fox PF (1994) Autolysis and proteolysis in different strains of starter bacteria during cheddar cheese ripening. *J Dairy Science* 61:249–262

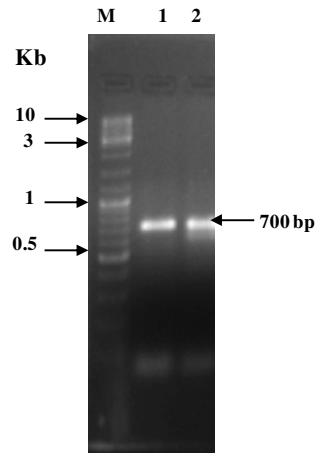
(A)



(B)



(C)



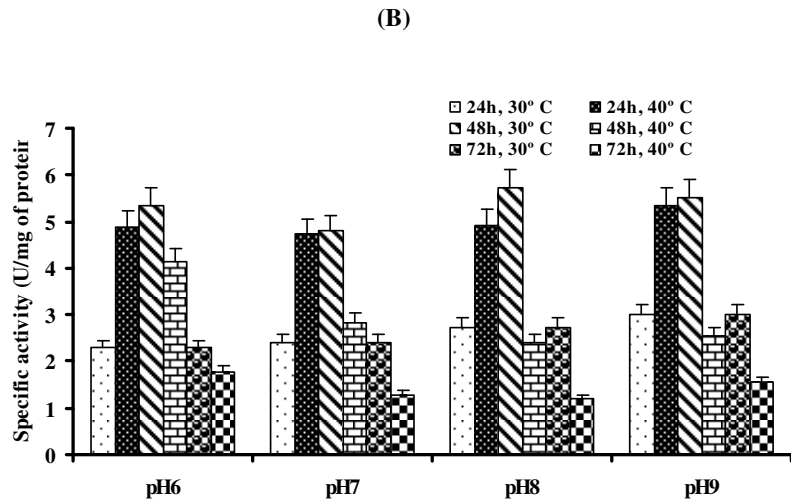
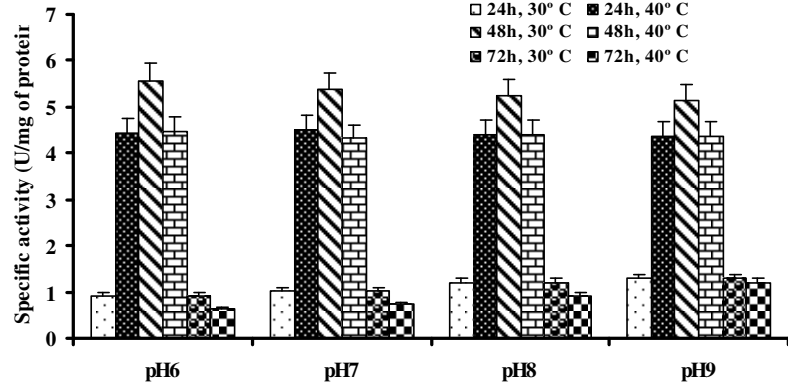
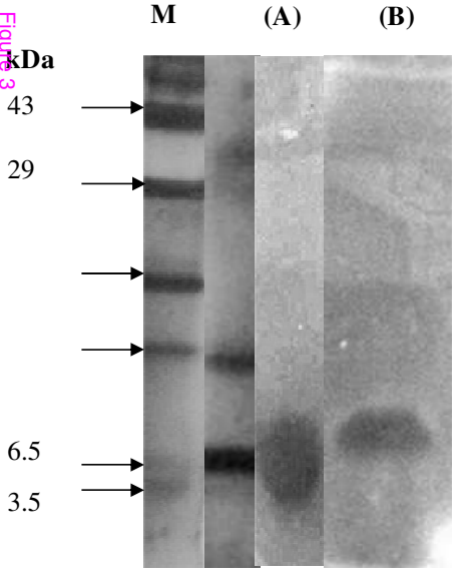
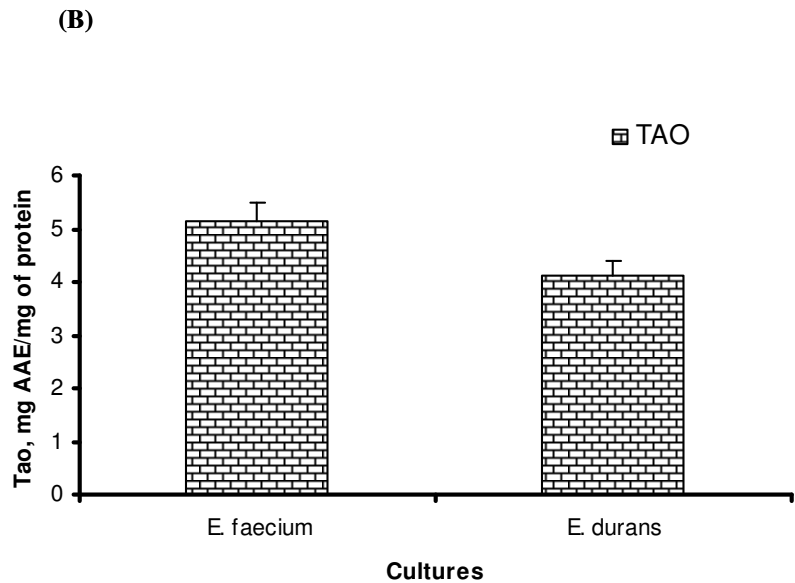
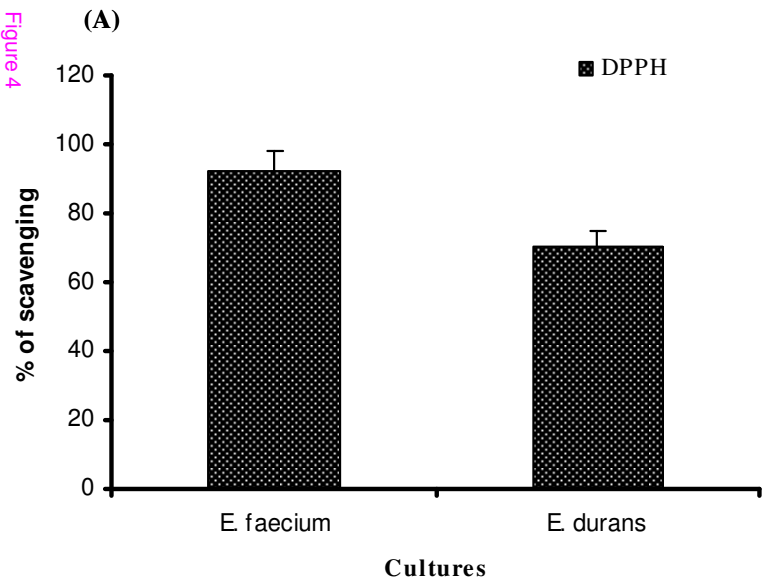


Figure 3





Additional files provided with this submission:

Additional file 1: Response to reviewer's comments.doc, 30K

<http://www.intaquares.com/imedia/1185128835773621/supp1.doc>