

## Original Research Article

### Isolation and production of bacteriocin by marine *Lactobacillus fermentum* SBS001

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## ABSTRACT

Some *Lactobacillus spp.* are used industrially for the production of Yoghurt, Cheese, sauerkraut, pickles, beer, wine, cider, kimichi, chocholate and other fermented foods, as well as animal foods, such as silage. The present study the bacteriocin from *Lactobacillus fermentum* isolated was protein in nature with antimicrobial effects on some clinically important food borne pathogens. This strain was isolated from the marine water sample and they were cultivated on MRS agar and identified using biochemical methods. The potential strain was selected by the antimicrobial well assay method. In the present study the optimal growth conditions for bacteriocin production were found to be at pH 9, 35°C, 2% salinity, glucose-1.0% as carbon source, 0.5% sodium nitrate as nitrogen source with 24hrs incubation. The protein bands were observed using SDS-PAGE and their molecular weight ranging from 78KDa. This reveals the potential application of bacteriocin produced by *L.fermentum* as a protective culture for the improvement of the microbial safety of fermented foods and reduction in food contamination which causes illness to human beings. The present study thus confirmed the possibility of using the strain *L. fermentum* as a biopreservative and a probiotic.

## Keywords

Marine  
*Lactobacillus fermentum*;  
optimization;  
antimicrobial activity;  
SDS-PAGE.

## Introduction

Bacteriocins are polypeptides, with bactericidal or bacteriostatic activity, against those bacteria which are closely related to the producer strain (Klaenhammer, 1998 and Tagg *et al.*, 1976). Bacteriocins are highly specific antibacterial proteins produced by strains of bacteria active mainly against some other strains of same or related species

(Gaur *et al.*, 2004). The bacteriocins produced by gram positive bacteria, in particular, the lactic acid bacteria display fairly broad inhibitory spectra with food preservative (Galvez *et al.*, 2008) and therapeutic (Jack *et al.*, 1995) potentials. Bacterial strains with antimicrobial activity play an important role in the food industry, agriculture and pharmaceutical industry. Different bacteriocin exhibits

different profile on food spoilage and pathogenic microorganisms. Therefore, they could be natural replacements for synthetic food preservatives. Bacteriocinogenic bacterial strains appear to be an excellent candidate for a friendly alternative since bacteriocin would be used as an antibiotic substitute (Joerger *et al.*, 2003), whereas bacteria would be a potential probiotic (Gillor *et al.*, 2008).

The use of bacteriocins or the microorganisms that produce them is attractive to the food industry in the face of increasing consumer demand for natural products and the growing concern about food borne diseases (Joshi *et al.*, 2006). Probiotics are live microorganisms that, when consumed in an adequate amount as part of the food, confer the health benefit on the host (FAO/WHO, 2001). An experimental focus on bacteriocin production by probiotic LAB strains has indicated that this potential might play a considerable role during *in vivo* interactions occurring in the human gastrointestinal tract, for instance towards *H. pylori* (Devuyst *et al.*, 2004; Avonts and Devuyst 2001 and Kim *et al.*, 2003).

Lactic acid bacteria (LAB) are among the best microbes, constituting a large group of beneficial bacteria that produce lactic acid from fermentable carbohydrates. LAB is Gram-positive, aero-tolerant anaerobes, non sporulating rods and/or cocci frequently occurring in chains. They are wide spread in nature and can be isolated from grains, green plants, fermenting vegetables, mucosal surface of animals, dairy and meat products (Lindgren and Dobogosz, 1990). The marine LAB could play a significant role in transformation of organic matter, especially in the surface of deep marine sediments. Marine LAB has

unique features which are, different from terrestrial LAB. The *Carnobacterium* species from the Antarctic lake are neutrophilic, growing best at pH 7.0. When this is grown in broth culture with pH 8.5, the final pH reduced to as low as 4.7-5.2. This low pH may exclude other bacteria present in seawater-associated alkaline environments (Spielmeyer *et al.*, 1993).

## Materials and Methods

### Collection of samples

Water sample was collected from Vellar estuary, Tamilnadu, India and they were serially diluted. 0.1ml from  $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$  dilutions were spread plated over the surface of the MRS agar plates. The plates were incubated at 37°C for three days. The colonies were observed.

### Antimicrobial activity against human pathogens

Nutrient broth was prepared and sterilized. Ten different human pathogens such as *S.aureus*, *Salmonella typhi*, *S.paratyphi*, *K.oxytoca*, *P.aeruginosa*, *E.coli*, *P.mirabilis*, *L. bulgaricus*, *V.cholerae* and *K.pneumoniae* were inoculated separately and kept for incubation. Nutrient plates were swabbed with 0.1 ml of different human pathogens. After swabbing, wells were punctured and cell free extracts of different strains of *Lactobacillus* was poured in to individual well and kept for incubation at 37°C. The plates were observed for a clear zone of inhibition and measured after 24 hrs. The potential *Lactobacillus* strain was selected based on the diameter of the zone of inhibition given in Table1. The potential strain was identified according to the method described by Buchanan *et al.*, (1974).

### Optimization for growth

The factors like pH, temperature, salinity and substrate concentration which were expected to influence the production of bacteriocin by the selected strain were optimized by selecting one parameter at a time. Effect of different pH on growth (i.e.) 6, 7, 8, 9, 10 and 11, different salinity 0.5%, 1%, 1.5%, 2% 2.5% and 3.0%, different substrates like carbon source, glucose (1.0%) and nitrogen source, peptone (0.5%) were checked for every 6hrs.

### Mass scale production

Mass scale production was carried out with optimized parameters like temperature-35°C, salinity-2%, Glucose-2%, peptone-2% were used for the mass scale maintained in the medium (250ml). Four such flasks were kept for incubation at 35°C in a shaker for 24hrs.

### Partial purification of bacteriocin

The cell free extract was collected by spun the tube at 15,000 rpm for 10min. The supernatant was precipitated with 80% ammonium sulphate. Dialysis was followed in a tubular cellulose membrane against 2L distilled water for 24hrs at 4°C. The partially purified bacteriocin was lyophilized in a Vertis lyophilizer and kept for further analysis.

### Protein Separation - SDS-PAGE- (Laemmli, 1973)

The proteins were separated by SDS-PAGE electrophoresis and size of polypeptide chains of given protein can be determined by comparing its

electrophoretic mobility in SDS-PAGE gel with mobility marker proteins of known molecular weight.

### Results and Discussion

Several types of bacteriocins from food-associated lactic acid bacteria have been identified and characterized, of which nisin, diplococcin, acidophilin, bulgarican, helveticins, lactacins, lactolin and plantaricins are the important bacteriocins. Of these, bacteriocin and nisin produced by *Lactococcus lactis* sp. lactis, has been the most extensively characterized. Nisin is the only bacteriocin commercially available. It has been reported that nisin is more active against Gram-positive bacteria, particularly the spore-formers. Other bacteriocins of *Lactobacilli* have been reported to be effective against closely related species of mesophilic *Lactobacillus* and therefore considered as potential natural food preservatives (Ray *et al.*, 2001).

The present investigation highlights the isolation, partial characterization and activity of bacteriocin produced by *Lactobacillus fermentum* SBS001. Marine samples seem to be a good source of lactic acid bacteria. The most potential strain was selected and used for further study. The physicochemical characterization of the strain revealed that it is *L. Fermentum* SBS001. *Lactobacillus fermentum* SBS001 based on its, morphological, physiological and biochemical characteristics. The strain was gram positive, non motile, non spore forming and rod shaped. It showed positive reaction in oxidase, fermentation in fructose, glucose and mannitol. It was catalase negative and negative in MR-VP test and nitrate reduction. It did not produce pigment. Karthikeyan and

Santhosh (2009) study revealed that *P. monodan* harbored LAB at the level of  $4.0 \times 10^5$  CFU/g in its gut, among strains tested; the most potential strain was selected and used for further study. The physicochemical characterization of the strain revealed that it was *L. plantarum*.

**Table. 1** Antimicrobial activity of *Lactobacillus fermentum*

Bacterial	zone of inhibition (mm)
<i>Staphylococcus aureus</i>	12
<i>Salmonella typhii</i>	10
<i>Salmonella paratyphi</i>	8
<i>Klebsiella oxytoca</i>	8
<i>Pseudomonas aeruginosa</i>	12
<i>Escherichia coli</i>	8
<i>Proteus mirabilis</i>	7
<i>Lactobacillus bulgaricus</i>	8
<i>Vibrio cholerae</i>	8
<i>Klebsiella pneumoniae</i>	7

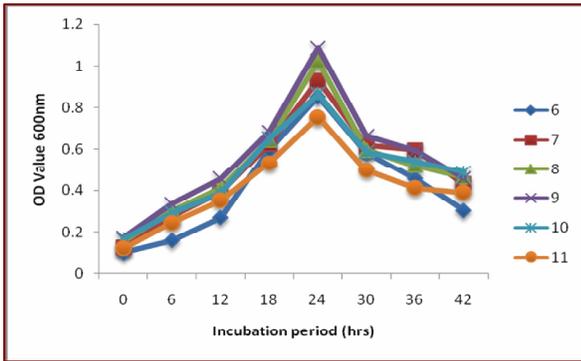
Bacteriocins are proteinaceous antibacterial compounds and exhibit bactericidal activity against species closely related to the producer *Lactobacillus* strains. Many bacteriocins are active against food-borne pathogens, especially against *Listeria monocytogenes*. Bacteriocins are heterogeneous and they are classified largely based on differences in their molecular weights. Some bacteriocins are peptides consisting of only 13 to 37 amino acids. Some small bacteriocins contain unusual amino acids originating from modifications of conventional amino acids after translation (Salminen *et al.*, 1996).

In the present study *Lactobacillus fermentum* SBS001 showed inhibitory activity (mm) to the pathogens in the order of *Staphylococcus aureus* (12), *P.aeruginosa*(12), *Salmonella typhi* (10), *S.paratyphi* (8), *K.oxytoca*, (8) *E.coli* (8), *L. Bulgaricus* (8), *V.cholerae* (8), *P.mirabilis* (7) and *K.pneumoniae* (7). Surprisingly all of them were found to be sensitive to *Lactobacillus fermentum* SBS001 (Table: 1). *L. fermentum* SBS001 showed maximum inhibitory activity to the human pathogens such as *Klebsiella oxytoca*, *Pseudomonas aeruginosa* and *E-coli* and minimum towards *S. paratyphi*, *Proteus mirabilis*, *Vibro cholerae* and *K.pneumoniae*.

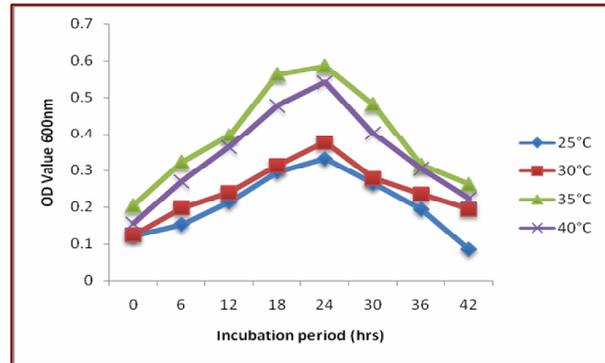
The study had proved the possibility of using this strain as a biopreservative or as a probiotic. The well-agar method of assay revealed the lactic acid bacteria *Lactobacillus fermentum* was inhibitory to bacterial pathogens. *L. fermentum* SBS001 was tested against ten human pathogens for antimicrobial activity by well assay or spot test method as described Karthikeyan and Santhosh (2009). They found that their *Lactobacillus* strain *L. acidophilus* was inhibitory to all the pathogens they tested except the *Vibrio cholerae*.

In the present study the optimal growth conditions for bacteriocin production were found to be at pH 9, 35°C, 2% salinity, glucose-1.0% as carbon source, 0.5% sodium nitrate as nitrogen source with 24hrs incubation (Fig. 1-5). karthikeyan and santhosh (2009) reported various physicochemical factors seemed to affect bacteriocin production as well as its activity. Maximum activity was noted at pH 5, temperature 40°C and 0.9 NaCl.

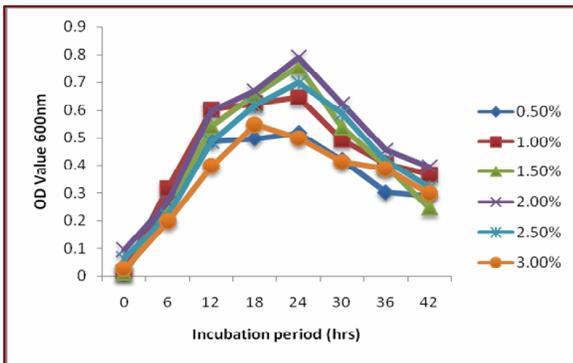
**Figure. 1** Effect of pH on growth



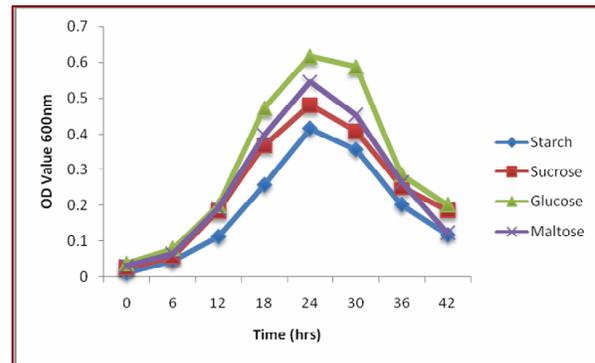
**Figure.3** Effect of Temperature on growth



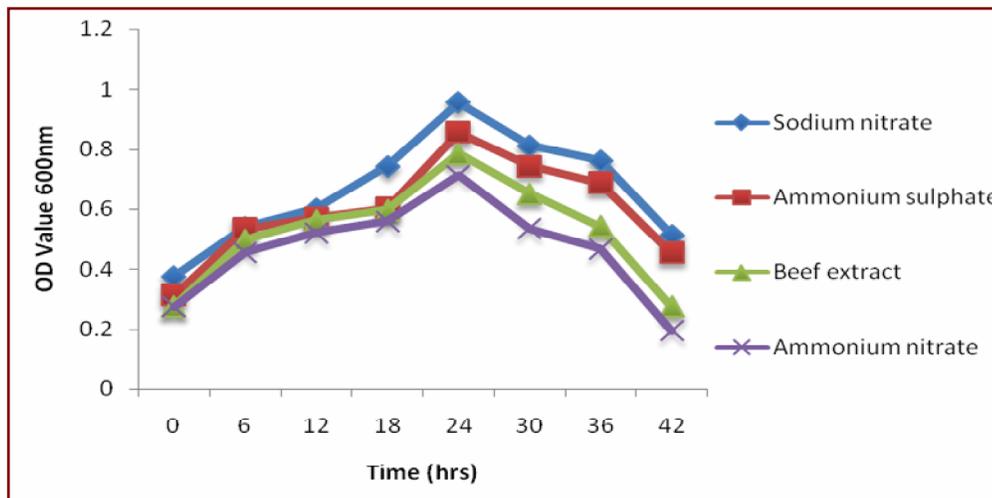
**Figure. 2** Effect of salinity on growth



**Figure.4** Effect of Carbon source on growth

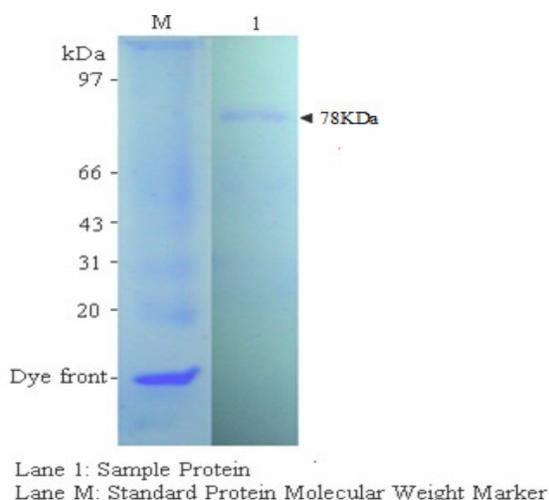


**Figure. 5** Effect of Nitrogen sources on growth



From the results proved that it can be used in fermented acidic foods like pickle or yogurt. MRS seemed to be more suitable medium compared to GP broth for the bacteriocin production and the bacteriocin secretion was started at 14 hrs culture, it might be a secondary metabolites. An earlier study by the senior author (Sarika, 2003) revealed that in *L. plantarum* MTCC1746, maximum bacteriocin production could be achieved by providing 1.5% yeast extract and 1.5% Tween-80. The addition of MgSO<sub>4</sub> could make a slight impact on the production of bacteriocin.

**Figure.6 Protein analysis of Bacteriocin**



In the present study the bacteriocin is a pure protein moiety which had a molecular weight of 78 KDa (Fig:6). Bacteriocin production was strongly dependent on pH, nutrient source and salinity and incubation temperature. Further purification is needed and whether both fractions are having inhibitory activity or not to be decided. Karthikeyan and Santhosh (2009) stated that the purified bacteriocin was purely protein moiety, because enzyme inhibitor study proved that the bacteriocin was only inhibited by protease and their molecular

weight of the protein was determined about 2.5 kDa.

The present study showed that the bacteriocin of *Lactobacillus fermentum* SBS001 is a protein with antimicrobial effects on some clinically important food borne pathogens. This reveals the potential application of bacteriocin produced by *L.fermentum* as a protective culture for the improvement of the microbial safety of fermented foods and reduction in food contamination which causes illness to human beings. The present study thus confirmed the possibility of using the strain *L. fermentum* as a biopreservative and a probiotic.

## References

- Avonts, L., and DeVuyst,L. 2001. Ribosomally synthesized antibacterial peptides in gram positive bacteria. *Curr. Drug. Targ.*, 3:107-122.
- DeVuyst, L., L. Avonts, P. Neysens, B. Hoste, M. Vancanneyt, J.Swings and Callewaert, R. 2004. The Lactobin A and amylovorin L47, encoding genes are identical and their distribution seems to be restricted to the species *Lactobacillus amylovorus* that is of interest for cereal fermentations. *Inter. J. Food Microbiol.*, 90: 93-106.
- Galvez, B., P. Arca, B. Mayo and Suarez, J. E. 2008. Detection, purification and characterization of plantaricin C, a bacteriocin produced by a *Lactobacillus plantarum* strains of dairy origin. *Appl. Environ. Microbiol.*, 60: 2158-2163.
- Gaur Y., K. Narayan, S. Chauhan and Ali, A. 2004. Bacteriocinogeny: concept, nomenclature, prevalence and application. *Indian Journal of Microbiology*, 44(1): 1-30.

- Gillor, Y., S. Jia, Q. Gao and Tan, Z. 2008. A novel bacteriocin with a broad inhibitory spectrum produced by *Lactobacillus sakei* C2, isolated from traditional chine fermented cabbage. Food control, 21: 76-81.
- Jack, R.W., J.R. Tagg and Ray, B. 1995. Bacteriocins of gram-positive bacteria. Microbiol. Rev., 59: 171-200.
- Joerger, R.D., S. Salminen and Arvilommi, H. 2003. Alternatives to Antibiotics: Bacteriocins, Antimicrobial Peptides and Bacteriophages. Poultry Sci., 82: 640-647.
- Joshi, R.W., J.R. Tagg and Ray, B. 2006. Bacteriocins of Gram positive bacteria. Microbiol. Rev., 59:171-200.
- Karthikeyan, V. and Santosh, S. W. 2009. Study of bacteriocin as a food preservative and the *Lactobacillus acidophilus* strain as probiotic. Pak. J. Nutr. 8(4): 335-340.
- Kim, B.V., S.F. Barefoot, R.F. Testin and Hayasaka, S.S. 2003. Detection and activity of plantaricinF, an antibacterial substance from *Lactobacillus plantarum* BF001 isolated from processed channel catfish. J. Food. Prot.57: 698-702
- Klaenhammer, K., 1998. Inhibition of *Listeria monocytogenes* in cold smoked salmon by addition of sakacin P and /or live *Lactobacillus sakei* cultures. Food. Microbiol., 18:431-439.
- Lindgern .S.E. and Dobrogosz, W.J. 1990. Antagonistic activities of lactic acid bacteria in food and feed fermentations. FEMS Microbiol. Rev. 87: 149-164.
- Ray, W., C. Santivararigkna, P. Jumriangrit, K. Sonomoto and Panyim,S. 2001. Isolation of nisin-producing *Lactococcus lactis* WN C20 strain fromnham, a traditional Thai fermented sausage. Intern. J. Food Microbiol., 81:137-145.
- Salminen, S., F. Sun, A. Vanghan and Sinderen, D.V. 1996. High-Throughput isolation of bacteriocin-producing Lactic Acid Bacteria, with potential application in the Brewing industry. Inst. Brew.Distill., 113(3):1121-523.
- Sarika, A.R., Schmitz, S., A. Hoffmann, B. Rudd and Bierbaum,G. 2010. Optimization analysis of Free and Immobilized bacteriocin *elaborated by Lactobacillus plantarum* MTCC B 1746 and *Lactococcus lactis* MTCC B 440, M.Sc. Dissertation, Manonmaniam Sundaranar Univ., Thirunelveli, Tamil Nadu, India, pp: 54.
- Spielmeyer, S., A. Hoffmann, B. Rudd and Bierbaum, G. 1993. The lantibiotic mersacidin is an auto-inducing peptide. Appl. Environ. Microbiol., 72:7270-7277.
- Tagg, J. R., Jones, R.J., H.M. Hassen, M. Zagovac and Brightwell, G. 2008. Isolation of lactic acid bacteria with inhibitory activity against pathogens and spoilage organisms associated with fresh meat. Food Microbiol., 25:228-234.