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Isolation and Partial Characterisation of Bacteriocin Produced By *Lactobacillus Fermentum*

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Abstract: *The present study was confined to the velar estuary in Cuddalore District, Tamil Nadu.Water samples were collected to isolate the bacteria. The collected samples were plated (MRS agar), incubated and the Bacterial colonies were identified. Effect of different ecological parameters such as pH (6-11), temperature (30-60ºC), salinity (0.5-3%), substrates (carbon &nitrogen source) on the growth of bacteria was also determined. Maximum bacterial growth was observed in pH 9, 35ºC temperature, 2% salinity, 1% glucose as carbon source and 0.5% sodium nitrate as nitrogen source after 24hrs of growth in liquid medium. The potential strain was selected by the antimicrobial well assay method. Lactobacillus fermentum (Bacteriocin) contains inhibitory activity against histamine producers 10 strains) . Antimicrobial activity was tested against 10 human pathogens. The protein bands were observed using SDS-PAGE and their molecular weight ranging from 78KDa.*

Keywords: *Growth characteristics - Effect of physical and chemical parameters, SDS-PAGE, Lactobacillus fermentum (Bacteriocin)*

I. 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).Bacteriocins were first discovered by A.Gratia in 1925. He was involved in the process of searching for ways to kill bacteria, which also resulted in the development of antibiotics and the discovery of bacteriophage, all within a span of a few years. He called his discovery a colicin because it killed *E.coli.* 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. Many bacterial species inhabit the ecological niches with a limited amount of nutrients. Because of this, many bacterial species produce a variety of antimicrobial substances, such as lactic acid, acetic acid, diacetyl, hydrogen peroxide and the other substances including enzymes, defective phages and lytic agents with potential importance for food fermentation and biopreservation (Lindgren and dobrogosz, 1990).

Bacteriocin production seems to be aimed to compete against other bacteria which are present in the same ecological niche (Barefoot et al., 1993 and Dykes, 1995 and Riley,1998). Different bacteriocin exhibits different profile on food spoilage and pathogenic microorganisms. Therefore, they could be natural replacements for synthetic food preservatives. Bacteriocin production has been reported to be affected by several factors including carbon and nitrogen sources and fermentation conditions, such as pH, temperature and agitation (Kim et al., 2006). 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).

Till the end of 1950, a very few of the food items were processed and packaged, processed and packaged foods were luxury item in colonial times but after 1960, these food items were in great demand globally due to growing urbanization, breakdown of large families in to nuclear families and increase in the number of working women. Chemical preservatives and other traditional barriers have been used in the food products to inhibit microbial growth which led to serious health diasasters, thus challenging the food scientist for providing safer and healthier food. Food preservation has become a major issue because food borne pathogens can cause havoc in preserved /fresh food items at high temperature, room temperature and even at low temperature (Sharma et al., 2006). However consumer demand for faster, healthier and ready –to-eat

products have strongly demanded the use of more natural preservatives instead of chemical preservative. Microbiologists around the world became interested in bacteriocin production the requirement of food preservation.

The following requirements should be fulfilled by any biopreservative to be used commercially (Holo et al., 2002; Kim and Bhowmik 1990 and Mauriello et al., 1999).

1. The biopreservative to be used should not be toxic.
2. It should be accepted by recognized authorities.
3. It should be economical to the industries using it.
4. The product in which the biopreservative is being used should not be affected by it, i.e. biopreservative should not show any deleterious effect toward the organoleptic properties of that product.
5. When used at relatively low concentrations it should show effectiveness.
6. The biopreservative should be sufficiently stable in storage.
7. It should not have any medicinal use.

Bacteriocins fulfil all the above requirements and hence are gaining popularity in the food industry day-by-day. Bacteriocins have been grouped into four main distinct classes (Klaenhammer, 1993).

Class I	Lantibiotics characterized by the presence of unusual thioether amino acid which are generated through translational modification.
Class II	Bacteriocin represent small(<10KD) heat stable, membrane active peptides.
Class II A	Subclass II A represented by *Listeria* active peptides which contain the N terminally located consensus sequence YGNGCXV where X is any amino acid.
Class II B	Representing portion complexes that require two different peptides for activity.
Subclass II C	Peptide whose exterrnalization into the growth medium of the producing bacterium is dependent on the general secretory pathway.
Class III	Bactteriocins belonging to class III consist of large(>30KD) heat labile protein.
Class IV	Represent complex bacteriocin that contain essential lipid, carbohydrate moieties in addition to a protein compared.

Kollath was the first to suggest, in 1953, the term "probiotics" to designate organic or inorganic substances that are essential to a healthy development of life (Kollath, 1953). 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 *invivo* interactions occurring in the human gastrointestinal tract, for instance towards *H. pylori* (De vuyst et al., 2004; Avonts and De vuyst 2001 and Kim et al., 2003).

The properties of *L.acidophilus* have been investigated in order to establish its specific role in the complex microbial equilibrium, both of man and higher animals. Among the *Lactobacillus* species, *L. acidophilus* strains have been extensively utilized as probiotic cultures in dairy and pharmaceutical products and numerous report have proved its ability to produce bacteriocin (De souza et al., 2005).

II. Materials And Methods

Collection of samples

Water samples were collected from Vellar estuary 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.

Identification of the potential strain

The strains were identified according to the method described by Buchanan *et al.,* 1974.The tests are given in Table1.

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 37^0C. The plates were observed for a clear zone of inhibition and measured after 24 h. The potential *Lactobacillus* strain was selected based on the diameter of the zone of inhibition given in Table2.

Antimicrobial activity against Histamine producers

Fish samples were collected from Chidambaram fish market. Samples were serially diluted and appropriate dilutions were plated on the mineral medium incorporated with filter sterilized histidine of 1g/100 ml and kept for at room temperature 28± for 24h .250 ml of nutrient agar was prepared, autoclaved and poured on the plates. It was swabbed with histamine producer colonies which was already available in the research laboratory. The wells were punctured on the plates and 0.1 ml of *Lactobacillus* culture filtrate was added and kept for incubation at 37^0c for three days.

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 pH on growth

Different pH (i.e.) 6, 7, 8, 9, 10, 11 were maintained in the medium. Growth and activity were assessed for every 6hrs.

Effect of Salinity on growth

Different salinity 0.5%, 1%, 1.5%, 2% 2.5% and 3.0% were maintained in the medium. Growth and activity were assessed for every 6hrs.

Effect of Substrates on growth

The carbon source, glucose (1.0%) and nitrogen source, peptone (0.5%) were maintained in the medium. Growth and activity were assessed for every 6hrs.

Mass scale culture for bacteriocin production in shake flask

The optimized conditions, temperature-35ºC, salinity-2%, Glucose-2%, peptone-2% were maintained in the medium (250ml). Four such flasks were kept for incubation at 35^0C in a shaker for 24hrs.

Ammonium sulphate precipitation and dialysis

The shake flasks kept for mass scale production were taken after 24hrs and centrifuged at 15,000 rpm for 10min. To the supernatant, the amount of ammonium sulphate required to give 80% saturation was added slowly with stirring. Dialysis was followed in a tubular cellulose membrane against 2L distilled water for 24hrs at 4°C.

Lyophylization

The partially purified bacteriocin was lyophilized in a Vertis lyophilizer and kept for further analysis.

SDS-PAGE (Protein profile)

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.

III. Result

Lactic acid bacteria were isolated from water samples, Vellar estuary using MRS agar. The colonies were creamy white, transparent and smooth round in shape. The selected strain was identified as *Lactobacillus fermentum* 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 (Table: 1).

Table: 1 Biochemical identification of *Lactobacillus fermentum*

Test	Result
Gram's staining	+, Rod
Motility	Non-motile
Morphological characteristics	Small, circular and smooth
Spore	Non-spore forming
Pigment	_
Bile esculin	_
Bile solubility	Insoluble
Indole	_
Methyl red	+
Voges Proskaur	_
Citrate utilization	+
Nitrate reduction	_
Growth on acetate	+
Ribose fermentation	+
Growth at 50ºC	_
Growth at 45ºC	+
pH 3	_
Production of H_2S	_
Production of H_2O_2	+

Antimicrobial activity was tested against ten major human pathogens such as *Staphylococcus aureus, Salmonella typhi, S.paratyphi, K.oxytoca, P.aeruginosa, E.coli, P.mirabilis, L. bulgaricus, V.cholerae and K.pneumoniae.* Surprisingly all of them were found to be sensitive to *Lactobacillus fermentum*. *Lactobacillus fermentum* showed inhibitory activity to the pathogens in the order of *P.aeruginosa, Staphylococcus aureus, K.oxytoca, L. bulgaricus, Salmonella typhi, E.coli, Vibrio cholerae, Salmonella paratyphi, P.mirabilis* and *K.pneumoniae*. (Table: 2)

Table 2: Antimicrobial activity of *Lactobacillus fermentum*

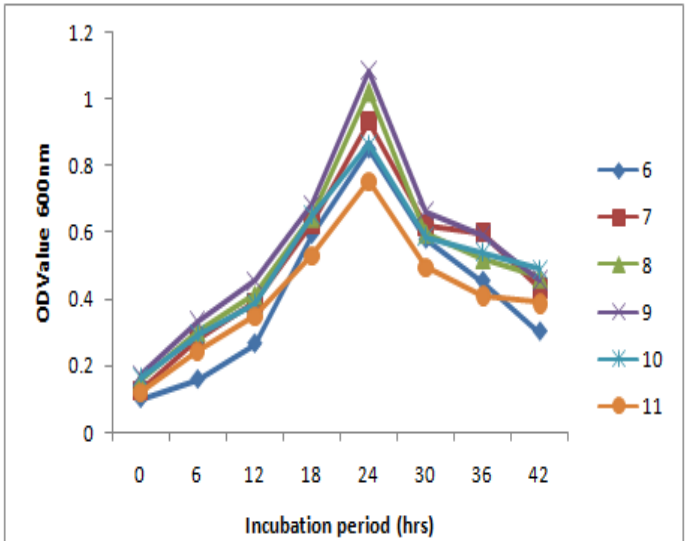
PATHOGENS TESTED	ZONE OF INHIBITION (mm)
Staphylococcus aureus	10
Salmonella typhii	8
Salmonella paratyphi	7
Klebsiella oxytoca	8
Pseudomonas aeroginosa	12
Escherichia coli	8
Proteus mirabilis	7
Lactobacillus bulgaricus	8
Vibrio cholera	8
Klebsiella pneumonia	7

In the present study histamine producers were isolated from fish samples obtained from the local market and checked with *Lactobacillus fermentum strain* for the inhibitory activity (Fig:3). 10 different histamine producing strains were inhibited by *Lactobacillus fermentum* (Table-3).

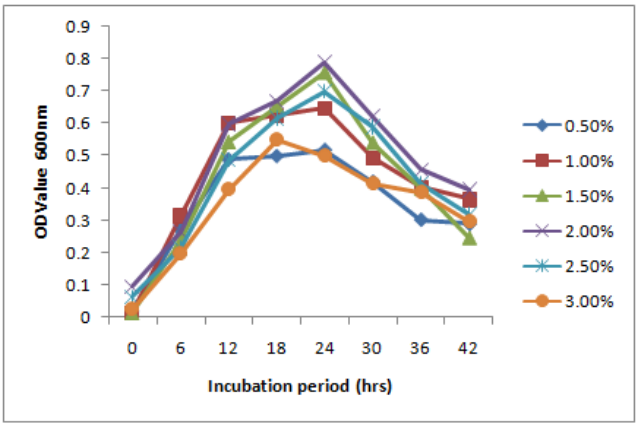
Table:3 Antimicrobial activity of *L. fermentum* culture filtrate against Histamine producers

Histamine Producers	Zone of inhibition (mm)
H1	9
H2	10
H3	5
H4	7
H5	11
H6	-
H7	3
H8	13
H9	5
H10	5

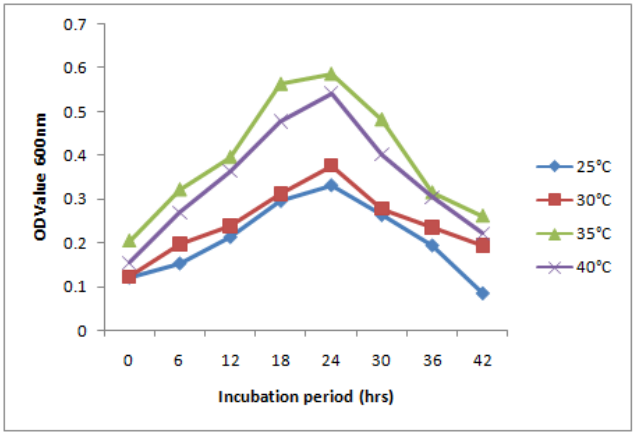
The optimal growth conditions for bacteriocin production were found to be at pH 9, 35ºC, 2% salinity, glucose-1.0% as carbon source, 0.5% sodium nitrate as nitrogen source with 24hrs incubation.



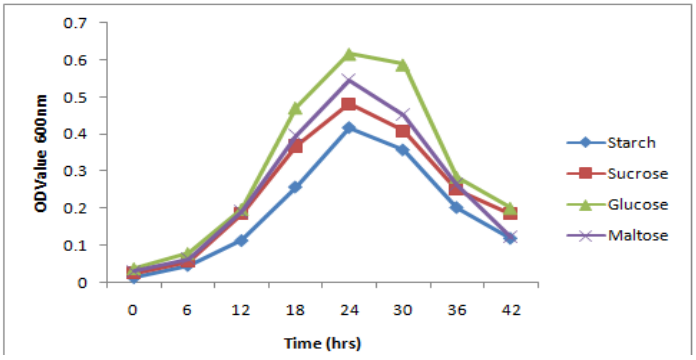
Effect of pH on growth



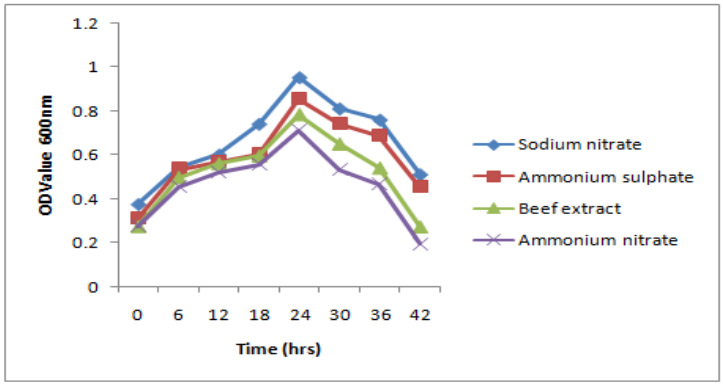
Effect of Substrates on growth



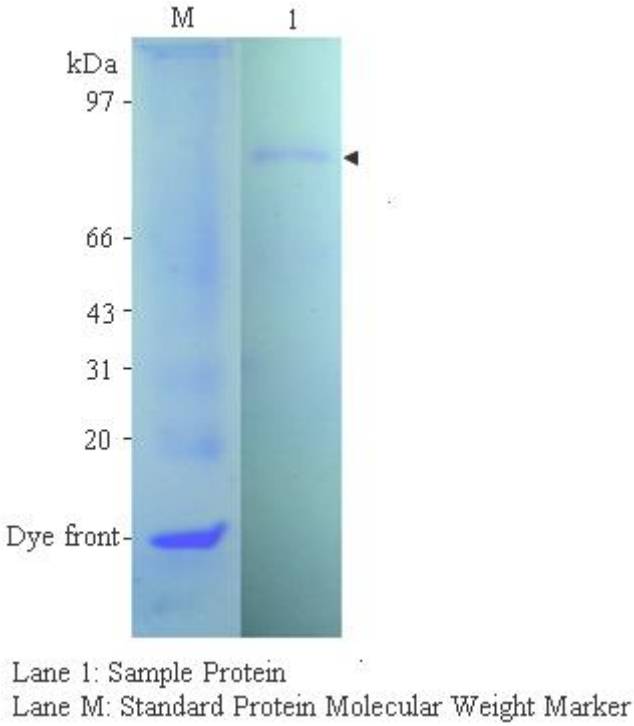
Effect of Temperature on growth



Effect of Carbon source on growth



Effect of Nitrogen sources on growth



Protein analysis of Bacteriocin

IV. Summary And Conclusions

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. Some *Lactobacillus spp.* and other lactic acid bacteria may possess potential therapeutic properties including anti-inflammatory and anti-cancer activites, as well as other features of interest. Research studies have demonstrated the protective effects of some strains of these bacteria for anti-tumor and anti-cancer effects. Reports also indicated that some cultures administrated to animals inhibited liver, colon, bladder and mammary tumors, highlighting potential systemic effects of probiotics with anti-neoplastic activities.

The present study showed that the bacteriocin of *Lactobacillus fermentum* is a protein with antimicrobial effects on some clinically important food borne pathogens and histamine producers. 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 confiemed the possibility of using the strain *L. fermentum* as a biopreservative and a probiotic.

In the present studies, *Lactobacillus fermentum* also contains the high protein content and antimicrobial activity. This strain was isolated from the marine sample and they were identified and cultivated using MRS agar. The potential strain was selected by the antimicrobial well assay method. *Lactobacillus fermentum* contains inhibitory activity against histamine producers also. The protein bands were observed using SDS-PAGE and their molecular weight ranging from 78KDa. The bacteriocin produced were rechecked.

V. References

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