WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.074
Volume 7, Issue 04, 209-221. Conference Article ISSN 2277–7105

STUDY OF BACTERIOCIN AND EPS PRODUCTION AND OPTIMIZATION USING NEW ISOLATES OF *LACTOBACILLUS* SPP.

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Article Received on
03 Jan. 2018,
Revised on 24 Jan. 2018,
Accepted on 13 Feb. 2018
DOI: 10.20959/wjpr20184-10775

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ABSTRACT

A total of six isolates of *Lactobacillus isolated* from different samples. These isolates were identified by phenotypic and biochemical methods. *Lactic acid bacteria* play an important role in food fermentation and preservation either as natural micro flora or as starter culture in the dairy industry. Lactic acid bacteria displays numerous antimicrobial activities some strains of lactic acid bacteria have importance in general health, providing a beneficial micro flora in the intestinal tract and are able to synthesize *Exopolysaccharides (EPS). Exopolysaccharides* produced by lactic acid bacteria have gained few years because of their contribution and texture of food products. The EPS was produced in simplified synthetic medium and extracted with two volumes of ethanol after 24 h of fermentation. The quantity of EPS produced by isolate was 1.27g. The dialyzed EPS was used in Nuclear Magnetic Resonance (NMR). This is mainly due to the production of organic acids but also of other compounds, such as *bacteriocins*. Bacteriocins are natural peptides varieties of bacteria for the purpose of killing other bacteria. In present study bacteriocin producing Lactobacillus species were isolated from different samples and bacteriocin was produced from Lactobacillus by fermentation. After isolation of bacteriocin it was tested for antimicrobial activity against five food spoilage causing and human pathogenic bacteria. In antimicrobial assay bacteriocin has showed as a strong antimicrobial agent against all five bacteria by forming zone of inhibition of 15mm, 12mm, 13mm, 19mm and 15mm respectively. This study revealed the possibility of using bacteriocin as bio preservative to control food spoilage causing bacteria Lactic acid bacteria products obtained with their aid are characterized by hygienic safety, storage stability and attractive sensory properties.

KEYWORDS: *Bacteriocins, EPS, lactobacillus and lactic acid bacteria.*

INTRODUCTION

Lactic acid bacteria are a group of Gram-positive bacteria united by a constellation of morphological, metabolic, physiological characteristics (Coeuret *et al*., 2003). They produce lactic acid either through homofermentative or heterofermentative pathway and are wide spread in nature and also found in human digestive system. Lactobacilli are considered especially as beneficial bacteria because they have their ability to break down proteins, carbohydrates and fats in food and help in absorption of necessary elements and nutrients such as minerals, aminoacids and vitamins required for the survival of humans and other animals. Lactic acid bacteria exert a strong antagonistic activity against many food-contaminating microorganisms as a result of the production of organic acids, hydrogen peroxide, diacetyl, inhibitory enzymes and bacteriocins (Piard and Desmazeaud, 1991).

The bacteriocins produced by LAB have been classified into four groups according to their biochemical characteristics. Because bacteriocins and their producers have potential applications as natural food preservatives, a number of bacteriocins have been identified and investigated (Chen & Hoover 2003). Generally, bacteriocins are peptides or proteins and different bacteriocins have different antimicrobial spectra. Bacteriocins are antimicrobial proteinaceous compounds that are inhibitory toward sensitive strains and are produced by both gram positive and gram negative bacteria (Tagg, J.R 1976). Bacteriocins are produced by bacteria and possess antibiotic properties, but bacteriocins are normally not termed antibiotics in order to avoid confusion and concern with therapeutic antibiotics that can potentially illicit allergic reactions in humans (Cleveland, J 2001).They are ribosomally synthesized polypeptide possessing bacteriocidal activity that are rapidly digested by proteases in the human digestive tract and relatively hydrophobic and heat stable (] Klaenhammer, T.R., 1998). The Bacteriocins (as colicins) were originally defined as bacteriocidal proteins characterized by lethal biosynthesis, a very narrow range of activity and adsorption to specific cell envelop receptors (Joerger, R.D 2000).

Exopolysaccharides (EPSs) produced by lactic acid bacteria (LAB) possess the possibility of replacing stabilizers and thickeners currently produced commercially by nonfood-grade bacteria. Over the past number of years, various studies have concentrated upon understanding the biochem- istry and genetics of EPS production in LAB, so that rational

strategies can be developed for the mprovement of EPS yield and the design of tailor-made polysaccharides.

Metabolic engineering strategies that target increasing metabolic flux to EPS should include EPS formation pathways (de Vos 1996). It has been suggested that a potential controlling factor in EPS biosynthesis is the availability of sugar nucleotides which are necessary for the construction of the polymers (Boelset al.2001).

This study revealed the possibility of using bacteriocin as bio preservative to control food spoilage causing bacteria Lactic acid bacteria products obtained with their aid are characterized by hygienic safety, storage stability and attractive sensory properties.

MATERIAL AND METHODS

Determination antibacterial activity

Collection of Samples

Sample like Fish, Curd, Lassi, Idli, Yakut, and Molasses were collected from different sources. Samples were taken to the laboratory for microbiological analysis.

Isolation and identification of bacteriocin producing bacteria

The bacteriocin producers from fish, curd, lassi, idli, yakult, and molasses were isolated by pour plate method technique using MRS(De Man, Rogosa and Sharpe agar) agar. After incubation for 24–48 h at 37 °C typical colonies were isolated and purified. The isolates were differentiated on the basis of their morphological, cultural and physiological characteristics such as oxidase test, utilization of citrate as a sole carbon source and catalase test and accordingly were tentatively, to the genus level. (Sharpe, 1979; Kandler and Weiss, 1986).

Agar Spot Assay Test

Lactic acid bacterial isolates were cultured in 5ml of MRS broth at 30° C for 16 hrs. Aliquots (2µl) of the culture were spotted onto agar plates containing 10ml of MRS medium. After 18 hrs at 30°C, the plates were overlaid with 5ml of the appropriate soft agar (1% agar) inoculated with the cell suspension of the indicator strain *Lactobacillus acidophilus* at a final concentration of 10 5 CFU/ml (Kilic *et al*., 1996). The plates were incubated at 37°C for 24-72 hrs, depending on the growth of the indicator strain and the appearance of inhibitory zones were observed. Inhibition was scored positive if the zone was wider than 2mm in diameter.

Agar-Well Diffusion Assay

The strains that were selected as potential bacteriocin producers were grown in MRS broth at 37ºC for 48 hrs. Cells were separated by centrifugation at 5000 rpm for 10 min at room temperature. Around 6mm diameter wells were made on preinoculated agar media and each well was inoculated with 100 µl of culture supernatant of bacteriocin producing *Lactobacillus* strains after neutralization with NaOH (Toba *et al*., 1991). Inhibitory activity was performed against certain Gram positive and Gram negative organisms: *Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa, Salmonella typhi,* Staphylococcus *aureus*.. Inhibition zones around the wells were measured and recorded.

Production of crude bacteriocin samples

Lactobacillus species was propagated in 1000 ml MRS broth and incubated at 24 hours at 37 ºC (anaerobically). For extraction of bacteriocin, a cell-free solution was obtained by centrifuging (10,000 rpm) for 20 min at 4 ºC then culture supernatant was adjusted to pH 7.0 by means of 1M NaOH to the antimicrobial effect, followed by filtration of the supernatant through a 0.2µl pore-size filter. The supernatant was dialysed for 24 h at 4 ºC and the cells were separated out supernatant was used as a crude bacteriocin.

Partial purification of bacteriocin

Ammonium Sulphate Precipitation: The crude bacteriocin samples were treated with solid ammonium sulphate75% saturation. The mixtures were stirred for 30mins in ice bucket and later centrifuged at 10,000 rpm for 20minutes (5ºC). The precipitates were re-suspended in 20 ml of 0.05 M potassium phosphate buffer (pH 7.0). Dialysis was followed in dialysis tubing and kept in stirrer for overnight incubation. Then 10ml the same buffer in dialysis tubing is kept for 18 h. Assay of the bacteriocin activity was carried out and was determined in both the precipitate and supernatant to know which one actually contain the bacteriocin.

Screening of isolates for antimicrobial activity

The inhibitory activity was screened by agar well diffusion assay. Overnight culture was inoculated in MRS broth and incubated at 24 hours at 37 ºC. Cells were removed by centrifugation at 10,000 rpm for 30 min at 4 ºC. The supernatant fluid was adjusted to pH-7 with 1N NaOH.Then test organisms were spread on Muller Hilton agar plate. Then the wells (5 mm) were cut into the agar plate by using a cork and 100 µl culture supernatant was placed into each well, plates were kept at cool temperature for 15minutes then incubated at 37 °C for

24 h (anareobically) the antimicrobial activity was determined by measuring the diameter of the inhibition zone around the wells. The bacterial isolate showing the widest zone of inhibition against the target microorganism.

Characterization of bacteriocin

Bacteriocin was checked for its temperature tolerance, pH stability and sensitivity to chloroform.

a) Sensitivity to heat

Culture supernatant was heated for 10 min at 40 ºC, 50 ºC, 60 ºC, 70 ºC, 80 ºC, 90 ºC, 100 ºC and agar well diffusion assay was performed to detect residual activity.

b) Sensitivity to different pH values

The pH of the culture supernatant was adjusted to 3, 4.5, 7.5 and 9.0 and then kept at room temperature for 4 h. Residual activity was determined by agar well diffusion method.

c) Sensitivity to chloroform

Chloroform was added to the supernatant at a final concentration of 0.5 mg/ml mix it well. The mixture was poured onto the culture plate well. The culture plates were examined after 18-24 h of incubation. The presence of inhibition zone around well both with and without chloroform were determined to be the effect of bacteriocin.

Optimization of culture condition to improve bacteriocin production

a) Optimization of medium composition

The influence of medium components on bacteriocin was evaluated by supplementing the media with glucose (1%), Tryptone (1%), NaCl (1%), Yeast extract (1%). The inoculated cultures were incubated for 24 hrs and the activity was assayed using agar well diffusion method.

b) Optimization of growth conditions

The effect of incubation period was studied by inoculating the producer organism into individual MRS medium and incubating at 37 ºC for period of 24, 30, 48 and 60 hours. Supernatants were determined by agar well diffusion method.

Determination of preservative effect of bacteriocin

The food products, *viz*. juice (water melon), pulp (grapes) were sterilized and inoculated with *Bacillus subtilis*, inoculated samples were recorded and bacteriocin supernatant at a concentration of 0.05 to 0.5 % was added. After 24 and 72 h, the plate count was recorded and compared with the control (without bacteriocin.)The partially purified bacteriocin from isolate was also tested for preservative effect against *B. subtilis* and clearly the preservative effect in juice, pulp increased with the increase in the concentration of bacteriocin. Maximum reduction of *B.subtilis population* was observed in juice as compare in pulp However, in control (without bacteriocin), no reduction was observed in the count of *B.subtilis*. The results revealed that microbial count decreased in pulp and was increased in juice and preservative effect of bacteriocin was seen in juice, it is a desirable characteristic of a bio preservative.

Production of EPS

Lactobacillus was grown in MRS broth for 24 h at 37ºC Disodium hydrogen phosphate was added by filtration through 0.2 µm membrane filter. Medium was inoculated and incubated for 24 h of fermentation under microaerophilic condition.

Isolation and estimation of Exopolysaccharides

The culture centrifuge for production media at 4 ºC at 8000 rpm for 30 minutes, collect the supernatant and mix it with equal volume of ice-cold chilled ethanol. Incubate at 4 ºC for 24 hrs.Centrifuge the above refrigerator solution at 4 ºC at 2500 rpm for 20 minutes.Resuspend the pellet in distilled water along with equal volume of ice-cold chilled ethanol, again centrifuge the solution at 4 ºC at 2500 rpm for 20 minutes. Dry the final pellet obtained at 60ºC and weighs it.

Determination of total carbohydrate content of *EPS* by phenol sulphuric acid method

To the dried pellet add 1 ml of 5% phenol and 5ml 69% concentration H2SO4,

Keep this mixture in ice water bath for 20 minutes. Take OD at 490nm.

Purification of EPS

EPS extracted from synthetic media may contain impurities (protein), which may interfere with EPS characterization. Hence, partial purification of EPS was done by dialysis. EPS was dialyzed against ethanol for 24 h at 37ºC. Partially purified EPS obtained from dialysis was

frozen at -20ºC in deep freezer.Frozen EPS was covered with parafilm and EPS for 1-2 days. Freeze dried EPS was used for further characterization.

Characterization of EPS

Morphology of freeze-dried EPS was determined by NMR analysis. Lyophilized powder of EPS was visualized under Nuclear Magnetic Resonance.

RESULT AND DISCUSSION

Isolation and identification of Lactobacillus spp.6 samples were isolated. The isolates were purified and characterized by morphological and biochemical tests according to these, include Gram positive, rod-shaped, non-motile.

Table 1: Antimicrobial activity of Bacteriocin against test organisms.

Sr.No	Name of test organism	Zone of inhibition formed by Bacteriocin (mm)
1.	*Escherichia coli*	15
2.	*Klebsiella pneumonia*	12
3.	*Pseudomonas aeruginosa*	13
4.	*Salmonella*	19
5.	*Staphylococcus aureus*	15

Characterization of bacteriocin

Table 2: Effect of temperature.

Temperature	Sensitivity
40 ºC	R
50 ºC	R
60 ºC	S
70 ºC	S
80 ºC	S
90 ºC	S
100 ºC	S

R=Resistance, S=Sensitive

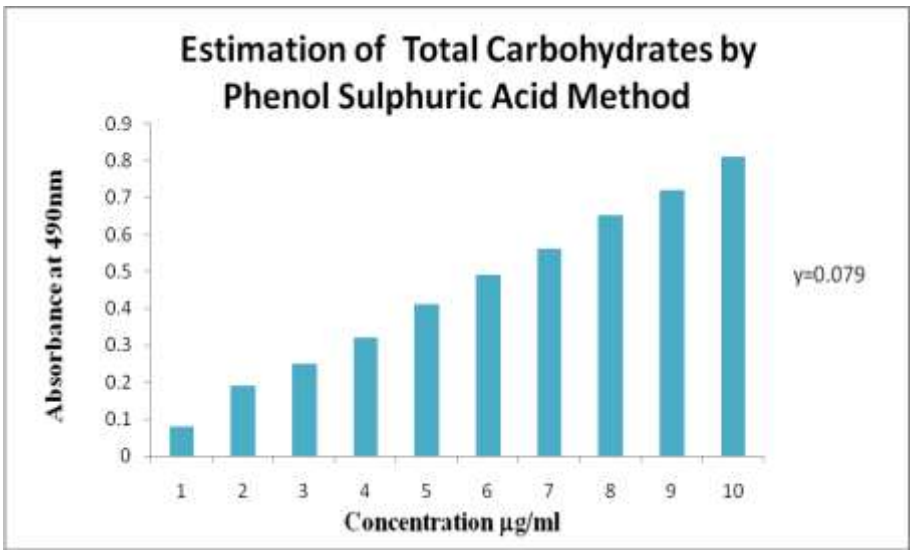
Table 3: Effect of Ph.

pH	Sensitivity
3	R
4.5	R
7.5	S
9	S

Table 4: Effect of chloroform.

Reagent	Sensitivity
Chloroform	Sensitive

Isolation and estimation of Exopolysaccharides



Calculation,

Absorbance of known sample =0.356

Y=mχ

0.356= (0.079)

χ=0.356/0.079=4.50

4.50µg/ml

Result The concentration of carbohydrate in known sample is 4.50µg/ml.

Characterization of EPS by NMR Analysis

EPS obtained from *Lactobacillus* in synthetic media was analysed by ^{1}H NMR as shown in figure 1. The freeze dried EPS was found to be a white powder and readily soluble in DMSO.

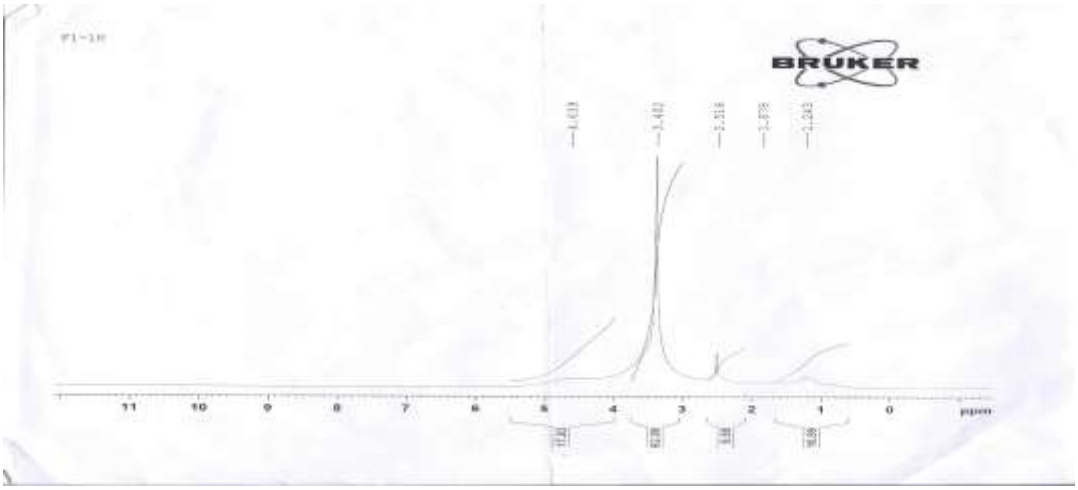


Figure 1: Analysis on 1H NMR of Fish-gut sample in DMSO.

All measurements were performed on a Bruker Advance III NMR spectrometer (Bruker, Karlsruhe,Germany) operating at 400.13 MHz for 1H observation, equipied with z axis gradient coil and automatic tuning-matching (ATM). Experiments were run in automation mode after loading samples on a Bruker Automatic Sample Changer, interfaced with the software Icon NMR (Bruker, Karlsruhe, Germany).

NMR peaks indicated the major functional groups and different types of protons in EPS. NMR peaks produced from *Lactobacillus* is given in figure. 1, the NMR peak at 1.243 ppm indicates the presence of hydroxyl group (OH) and methylene (CH2) and CH stretching was observed at 4.639 cm.Thus the NMR peaks reveals that EPS is complex polysaccharide containing different functional groups. Textural properties of EPS are contributed by its structure beside its quantity. In conclusion it can be said that studies on microbial exopolysaccharide are gaining importance especially from *Lactic acid bacteria*; thus EPS found to be a novel heteroplysaccharide.

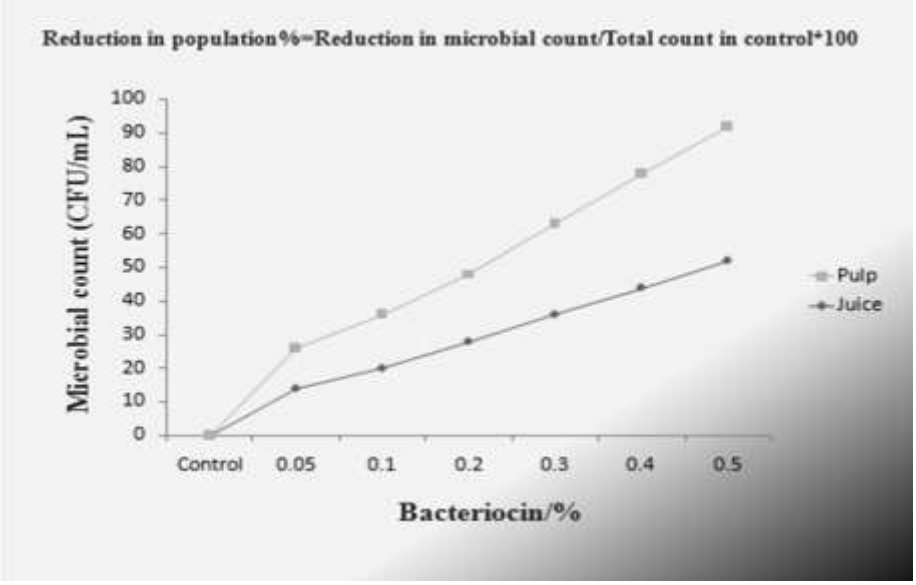
Determination of preservative effect of bacteriocin

The food products, *viz*. juice (water melon), pulp (grapes) were sterilized and inoculated with *Bacillus subtilis*, inoculated samples were recorded and bacteriocin supernatant at a concentration of 0.05 to 0.5 % was added.

Table 7: Preservative effect of partially purified bacteriocin From *Lactobacillus*, isolate in juice and pulp against *Bacillus subtilis*.

	Preservative effect against *B.subtilis**/%	
(bacteriocin)/%	Juice	pulp
Control	0	0
0.05	14	12
0.1	20	16
0.2	28	20
0.3	36	27
0.4	44	34
0.5	52	40

Maximum reduction of *B.subtilis population* was observed in juice as compare in pulp However, in control (without bacteriocin), no reduction was observed in the count of *B.subtilis*. The results revealed that microbial count decreased in pulp and was increased in juice and preservative effect of bacteriocin was seen in juice, it is a desirable characteristic of a bio preservative.



DISCUSSION AND CONCLUSION

Six isolates that had the characteristics of *lactic acid bacteria* were obtained. The isolates were initially differentiated on the basis of their cultural and morphological studies after which they were subjected to various physiological and biochemical tests.Detection of antimicrobial activity, the result of present study shows the highest inhibitory activity observed was against *E.coli, Klebsiella pneumonia, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella,* shows clear zone of inhibition[Table 3].(Ha, Duk-Mo*, Dong-Soo Cha and Seung-Guk Han in 1994).

Our results suggested that bacteriocin producing Lactobacillius naturally occurred and survive in fish gut. This also supported by Adenike and Olalekan (2009).who reported the isolation of 44 strains of *Lactobacillus* from the gut contains of African catfish(Clarias gariepinus).

The results revealed that microbial count decreased in pulp and was increased in juice and preservative effect of bacteriocin was seen in juice, it is a desirable characteristic of a bio preservative. Maximum reduction of *B.subtilis* population was observed in juice as compare in pulp However, in control (without bacteriocin), no reduction was observed in the count of *B.subtilis*[Table 7]. V. K. Joshi et al (2006). Exopolysaccharides(V.K.Joshi et al (2006))study on production, purification, stability and efficacy of bacteriocin. In our study the total no. of EPS produce is strongly influence and shows the activity of carbohydrates.

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