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Isolation of Probiotic Bacteria and Optimization of Physical and Nutrition Parameters for Bacteriocin Production

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A B S T R A C T

The aim of this study is to analyze probiotic properties of isolated lactic acid bacteria from human breast milk. Identification of lactic acid bacterial (LAB) was done by Gram's staining and catalase test and further confirmation was based on morphological, cultural, physiological and different biochemical tests. The isolated strain was identified after different biochemical analysis which was also showed reliable probiotic properties. These isolates were examined for further probiotic properties including tolerance to bile salt and resistance to low PH, antimicrobial activity. Probiotics are supposed to those bacteria which have beneficial effects for the host. The bacteriocin producing strains requires specific nutritional and cultural conditions for the growth and the metabolic production because the optimum growth will produce the maximum amount of metabolites- Optimization of bacteriocin production, the maximum culture density was found to be observed with starch (2.8mg/ml). Meat extract has shown the maximum cell mass among the tested nitrogen components. The bacteriocin inhibitory activity was also found to be optimum at 1% NaCl salt concentration. The bacteriocin also stable against wide range of pH and temperature.

Introduction

Probiotics, as defined by the Food and Agriculture Organization of United Nations (FAO) and World Health Organization (WHO) in 2001, comprise live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host. Probiotic products include different enzymes, vitamins, capsules or tablets and some fermented foods that contain microorganisms which have beneficial effects on the health of host. They can contain one or several species of probiotic bacteria. They are just used as health supporting products. The oral consumption of probiotic microorganisms produces a protective effect on the gut flora (Gismondo, *et al.*, 1999, Çakır 2003, Quwehand 1999). Bacteriocins are ribosomally synthesised antimicrobial peptides produced by bacteria which have bactericidal and bacteriostatic activity against other similar and dissimilar microbials. More than 99% of bacteria can produce at least one bacteriocin, most of them are not well-known (Riley and Wertz *et al.*, 2002). Bacteriocins are proteinaceous compounds that help to destroy closely related bacteria but also have

an action across the species. Many current studies focus that bacteriocin production is not limited by Gram-negative bacteria. Gram-positive lactic acid bacteria, a group of phylogenetically dissimilar microorganisms considered by some morphological, metabolic and physiological properties, comprise an resource for various bacteriocins that have a great possibility for industrial or medicinal applications due to the GRAS score (generally recognized as safe bacteria) of lactic acid bacteria (Abriouel *et al.,* 2007). In addition, the bacteriocins from Gram-positive bacteria show a special inhibitory effect that is not directed against only bacteria within the same species but also against another species. Thus, the bacteriocins produced by Gram-positive bacteria possess a broader range of susceptible organism

The inhibiting and killing capacity of bacteriocin is considered as a positive method for maintain the population and decreasing the number of challengers to achieve more nutrients and living spaces in that environment. Different other antibiotics, bacteriocins are secondary metabolites and very sensitive to protease enzymes like trypsin. These are present in human beings and also therefore harmless to human body. Many bacteriocins host range is narrow, and is likely to be most effective against related bacteria with nutritive demands for the same uncommon resources (Deegan *et al.,* 2006). Bacteriocins produced by some lactic acid bacteria, eg: nisin, salvarin, pediocinetc inhibit both closely related species and also food-borne pathogens such as *Listeria monocytogenes, E.coli, Vibrio cholera, S.aureu*s etc. and many other spoilage bacteria (Tagg and Dajani *et al.,* 1976). So that, bacteriocins are involved for use as natural food preservatives in juices, cheeses in recent years, this led to the discovery of ever increasing possible sources of these protein inhibitors.

Materials and Methods

Isolation of lactic acid bacteria from human breast milk

The isolation was done with human breast milk. For milk, samples were collected from the mother of 3rd day old kid. The samples were collected in sterile bottle and stored on ice until delivery to the laboratory. Once delivered to the laboratory, they were taken to the procedure for isolation. Pour plate technique was used to isolate the organisms. Samples were used directly and also diluted to 10^{-1}, 10^{-2} and 10^{-3} using sterile distilled water. 1mL of the samples and dilutions were plated into MRS agar (pH- 6.3). The plates were incubated at 37^0C for 3 days under aerobic conditions. After incubation, individual colonies were selected and transferred into sterile broth mediums. The following step is purifying the selected colonies with streak plate technique. The isolates were examined according to their colony morphology, catalase reaction and Grams reaction.

Identification and probiotic properties of isolated organism

Gram staining

The cultures were grown in appropriate mediums at 30^0C for 48 hour under aerobic conditions. After incubation gram staining procedure was applied. Then under light microscopy gram positives and purified isolates were determined.

Catalase test

Catalase test was performed to isolates in order to see their catalase reactions. For this purpose, overnight cultures of isolates were grown on MRS agar at suitable conditions. After 24 hour 3% hydrogen peroxide solution was dropped onto randomly chosen colony.

The isolates which did not form gas bubbles, since chose. Since, LAB is known as catalase negative.

For the determination of probiotic properties of isolates three major selection criteria were choosed

Resistance to pH
Tolerance against bile salt and
The antimicrobial activity

Resistance to low pH

Resistance to pH 3 is often used invitro assays to determine the resistance to stomach pH. For this purpose, active cultures (incubated for 16-18 hours) were used. Cells were harvested by centrifugation for 10 minute at 500 rpm and 4^{o}C. Pellets were washed out washed in phosphate – saline buffer (PBS) at pH7.2. Then cell pellets were re-suspended in PBS (pH 3) and incubated at 37^{o}C. Viable microorganisms were enumerated at the 0, 1, 2, 3 and 4 hours with pour plate techniques. Appropriate dilutions were done and plates were incubated at 37^{o}C under aerobic conditions for 48 hours. Also growth was monitored at OD_{620}. At 620nm and were expressed as Colony forming units.

Tolerance against bile salt

MRS medium containing 0.3% bile (oxide) was inoculated with active cultures (incubated for 16-18 hours). During the incubation for 4 hour, viable colonies were enumerated for every hour with pour plate technique and also growth was monitored at OD_{620}.

Antimicrobial activity

Modified agar well diffusion method was used to detect antimicrobial activities of cell free supernatant (CFS) produced from the isolates. Antibacterial activity was determined against, *Vibrio parahaemolytics, Listeria monocytogens, Vibrio cholera*. All of LAB isolates were incubated for 48 hour at 37^{0}C. After incubation cells were removed by centrifugation.

The indicator organism is inoculated in nutrient broth and incubated at 37^{0}C for 5- 6 hours. The incubated organisms swabbed on to the MHA (Muller – Hinton Agar) plates using swab and the CFS (Cell Free Supernatants) was used as antimicrobial agents. Using sterile tips the CFS was poured into the well of about 50µL and kept for incubation at 37^{0}C for 24 hours. Antimicrobial activity was evaluated by measuring zone of inhibition against the test organisms

Probiotic bacteria-optimisation of nutritional factors for bacteriocin production

Bacterial culture of probiotic organism was used for evaluating effects of physical and nutritional parameters on growth and bacteriocins production. The bacterial culture was inoculated on MRS broth containing – Protease peptone, beef extract, yeast extract, dextrose, tween 80, ammonium citrate, sodium acetate, magnesium sulphate, manganese sulphate, di potassium phosphate. pH of the media is adjusted to 6.3.

MRS broth was used for antimicrobial metabolite production from probiotic bacteria, 500mL of conical flasks each containing 200 ml MRS broth autoclaved at 121^{0}C for 15 minutes and inoculated with colony of a probiotic isolate grown on MRS agar. The inoculated flasks were incubated at 30^{0}C for 2-3 days under stationary condition. Then centrifuged at 100 rpm for 10 min. Antimicrobial activity of culture supernatant (100µl/well) and broth (100µl/well) was tested by agar well diffusion method (Ram *et al.*, 2013).

Quantification of cell growth

The cell growth at each set of experiment was monitored by measuring the optical density at 620 nm by spectrophotometer. The samples were withdrawn at the required time of interval to measure the optical density.

Quantification of protein

Total protein of the cell free supernatant was determined by the method of Lowery *et al.,* (1951) using Bovine Serum Albumin (BSA) as standard. The concentration of protein was calculated from the absorbance at 660 nm. The phenolic group of tyrosine and tryptophan residues reacts with the Folin – Ciocalteau reagent and gives a blue purple colour complex, having a maximum absorption at 540 nm. The intensity of colour is a direct function of the quantity of these amino acids present in protein.

Optimization of physical and nutrition parameters for bacteriocin production

Effect of carbon sources on cell growth and bacteriocin production

In order to evaluate the effect of different carbon sources on growth and bacteriocin production by Probiotic organism, 2% dextrose, fructose, sucrose, starch and lactose were supplemented in MRS broth. Each flask was incubated at 30^0C for 48 hours. The cell biomass and bacteriocins activities were determined as described previously (De Vuyst, 1995; Nelson *et al.,* 2001).

Effect of nitrogen sources on cell growth and bacteriocin production

The effect of different nitrogen on growth and bacteriocins yield of Probiotic organism was evaluated in MRS broth supplemented with 2% nitrogenous compound. The selected nitrogen sources were, meat extract, yeast extract, tryptone and bacteriological peptone. Each flask was incubated at 30^0C for 48 hour. The cell biomass and bacteriocin activity were determined as described previously (Verellen *et al.,* 1998).

Effect of salt concentration on cell growth and bacteriocin activity

To evaluate the effect of salt on cell mass and bacteriocins production, Probiotic organism was inoculated into 100mL MRS broth (pH – 6.3) supplemented with 1%, 2%, 3% and 4% NaCl salt concentration incubated in 30^0C for 48 hours. The cell density, bacteriocin activity, and protein concentration were determined as described previously (Verellen *et al.,* 1998).

Effect of temperature on cell growth and antibacterial activity

The influence of temperature on cell growth and bacteriocins activity of Probiotic organism was determined by MRS media. The cells were grown at a set of temperature 15^0, 30^0, 37^0 and 45^0C for 48 hours were incubated. Biomass, protein concentration and bacteriocins activity were determined by spectrophotometer (Verellen *et al.,* 1998; Graciella *et al.,* 1995; Juarez *et al.,* 2002).

Effect of pH on cell growth and bacteriocin activity

The influence of PH on cell growth and bacteriocins activity of Probiotic organism was determined by MRS media. The cells were grown at a set of PH 5,6,7,8 and were incubated at 30^0 c for 48 hours.

Biomass, protein concentration and bacteriocins activity were determined by spectrophotometer (Verellen *et al.,* 1998; Graciella *et al.,* 1995; Juarez *et al.,* 2002).

Temperature Sensitivity

To test the temperature sensitivity, the 48 hour culture supernatant was heated for 10 minutes at 60^0 C, 70 ^{0}C, 80^0 C and 90^0 C, for that the cell free culture supernatants of the probiotic strains were chosen for the antibacterial activity test.

The sensitivity of bacteriocins at different temperature and its bacteriocins antibacterial activity was detected by agar well diffusion method against sensitivity of bacteriocins to. *V. cholera, V. parahemolytics and L. monocytogens* (Ogunbanwo *et al.,* 2003)

Results and Discussion

Lactic acid bacteria were isolated from MRS medium at 37^0C. The catalase negative isolates were selected.

A catalase positive bacteria was also observed and they are excluded. From milk one catalase negative isolates were observed. Six of them found to be gram positive, catalase positive cocci.

Probiotic properties

Resistance to low pH

After incubation, optical density of the sample was measured at 620nm and viable cell count was also determined as colony forming unit.

From which it is clear that the isolate was able to survive in pH 3 for 4 hours. A significant increase in O.D value was observed during the interval. Hence it was concluded that the LAB isolate was tolerant to low pH.

Tolerance againt bile salt

The viable cells were determined by measuring O.D found to tolerate bile salt

Results of antimicrobial activity

The strains were examined according to their antimicrobial activity. For this purpose, strains were detected against the indicator microorganisms, *Vibrio cholera, Vibrio parahemolytics, Listeria monocytogens.*

The diameter of the inhibition zones showed that the isolates have antibacterial effect on the indicator microorganisms. The tests were done and the diameter of both crude and supernatants were obtained. For *Vibrio parahaemolytics*, the isolates showed large inhibition zone

In this study, Total 7 organisms were isolated from human breast milk. From that, C2 strain from human milk were selected after original characterisation and it is gram positive cocci and catalase negative and these probiotic bacteria is mainly used for further studies.

In this study the isolated strain showed the acid tolerance at pH 3 and the bile salt tolerance at 0.3%. Before reaching the intestinal tract, probiotic bacteria, must first survive transit through the stomach where the pH can be as low as 1.5 to 2. Bile salts are synthesised in liver from cholesterol and are secreted from the gall bladder into the duodenum. Physiological concentration of human bile from 0.1% - 0.3%

The isolated probiotic from human breast milk were strong antimicrobial activity against most of the tested enteric pathogens. This may be due to the production of bacteriocin or antibacterial compound. Antimicrobial activity of lactobacilli may be due to organic acids, hydrogen peroxide, bacteriocin or other inhibitory substances from metabolites (Todorov *et al.,* 2010). In this study C2 showed the strongest antagonistic potential against *Vibrio cholera, Listeria monocytogenes* and *Vibrio parahaemolyticus* (Table 1–10).

Table.1

SAMPLES	ISOLATES	GRAM STAINING	CATALASE TEST	COLONY MORPHOLOGY
milk	C1	+ve, cocci	-ve	Cream colour, pinpoint transparent colonies
	C2	+ve,cocci	-ve	Cream colour, small round colonies
	C3	+ve, cocci	+ve	White colour, small colonies
	C4	+ve, cocci	+ve	Small, round, transparent colonies
	C5	+ve, cocci	+ve	Cream colour, large round colonies
	C6	+ve, cocci	+ve	Small, white, opaque colonies
	C7	+ve, cocci	+ve	Small, white colour, flat colonies

Table.2

HOURS	OD at 620nm	CFU per ml
0th hour	0.532	TNTC
1st hour	0.541	TNTC
2nd hour	0.567	TNTC
3rd hour	0.571	TNTC
4th hour	0.542	TNTC

Table.3

HOURS	O.D at 620 nm	CFU per ml
0th hour	0.025	TNTC
1st hour	0.075	TNTC
2nd hour	0.192	TNTC
3rd hour	0.487	TNTC
4th hour	1.001	TNTC

Table.4

ORGANISMS	ZONE OF INHIBITION	
	CRUDE	SUPERNATENT
Vibrio parahaemolytics	16mm	16mm
Listeria monocytogens	13mm	14mm
Vibrio cholerae	12mm	13mm

Table.5

CARBON SOURCES	Mg/mL	ANTI - BACTERIAL ACTIVITY (Against - pathogenic organisms)			CELL GROWTH
	O.D. at 540nm	*V. cholera*	*V. parahaemolytics*	*L. monocytogens*	O.D. at 620nm
Fructose	4.723	10mm	16mm	15mm	1.508
Sucrose	4.949	12mm	19mm	20	1.743
Lactose	4.811	13mm	20mm	12mm	1.702
Starch	4.789	11mm	18mm	15mm	2.846

Table.6 Effect and influence of nitrogen sources on growth and bacteriocin production

NTROGEN SOURCES	Mg/mL	ANTI - BACTERIAL ACTIVITY (Against - pathogenic organisms)			O.D. at 620nm
	O.D. at 540nm	*V. cholera*	*V. parahaemolytics*	*L. monocytogens*	O.D. at 620nm
Yeast extract	4.566	Nil	17mm	22mm	2.149
Meat extract	4.947	Nil	28mm	22mm	2.939
Tryptone	4.833	Nil	20mm	21mm	2.357
Peptone	4.850	Nil	18mm	27mm	2.275

Table.7 Effect and influence of NaCl concentration on growth and bacteriocin production

NaCl CONCENTRTION	Mg/mL	ANTI - BACTERIAL ACTIVITY (Against - pathogenic organisms)			CELL GROWTH
		V. cholera	*V. parahaemolytics*	*L. monocytogens*	O.D. at 620nm
1 %	4.069	Nil	15mm	13mm	1.615
2%	3.909	Nil	14mm	17mm	1.476
3%	3.924	Nil	18mm	18mm	1.425
4%	3.918	Nil	14mm	16mm	1.381

Table.8 Effect and influence of temperatures on growth and bacteriocin production

DIFFERENT TEMPERATURE CONCENTRTION	Mg/mL	ANTI - BACTERIAL ACTIVITY (Against - pathogenic organisms)			CELL GROWTH
		V. cholera	*V. parahaemolytics*	*L. monocytogens*	O.D. at 620nm
150C	3.984	12mm	14mm	12mm	1.660
300C	4..550	14mm	15mm	14mm	1.812
370C	3.993	13mm	10mm	10mm	1.762
450C	4..037	10mm	10mm	11mm	1.250

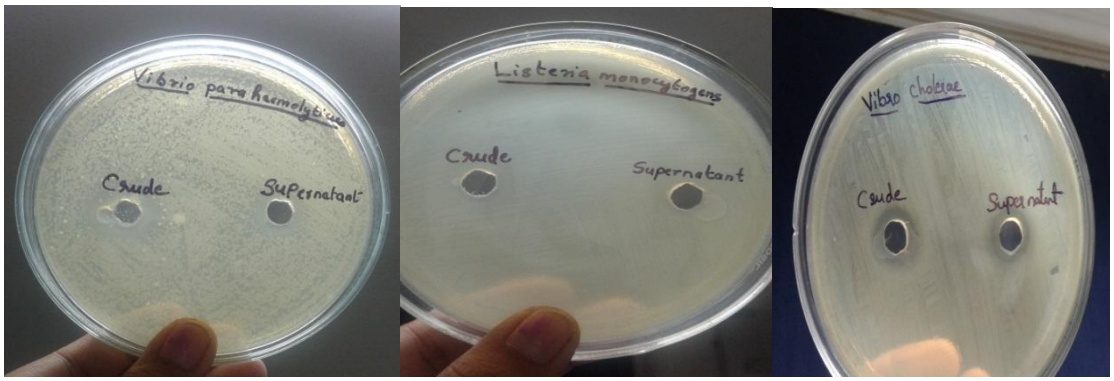
Table.9 Effect and influence of pH on growth and bacteriocin production

DIFFERENT pH CONCENTRTION	Mg/mL	ANTI - BACTERIAL ACTIVITY (Against - pathogenic organisms)			CELL GROWTH
		V. cholera	*V. parahaemolytics*	*L. monocytogens*	O.D.at 620nm
pH 5	4.358	Nil	11mm	18mm	0.760
pH 6	5.206	Nil	14mm	15mm	2.01
pH 7	4.844	Nil	12mm	16mm	2.030
pH 8	4.075	Nil	10mm	13mm	1.467

Table.10 Temperature sensitivity

DIFFERENT TEMPERATURE	ANTI - BACTERIAL ACTIVITY (Against -pathogenic organisms)		
	V. cholera	*V. parahaemolytics*	*L. monocytogens*
600C	Nil	15mm	10mm
700C	Nil	20mm	12mm
800C	Nil	18mm	13mm
900C	Nil	18mm	13mm

Effect and influence of carbon sources on growth and bacteriocin production



Obadina *et al.*, (2006) also reported that fermentation process, which involved *L. plantarum*, had a broad antimicrobial inhibitory spectrum, against *Salmonella typhi, E.coli, and S.aureus*. Hence, isolated probiotics can be useful to prevent enteric infections such as diarrhoea, dysentery, typhoid, food poisoning etc.

The probiotic strains produce many inhibitory substances with bactericidal or a bacteriolytic activity. Main objective of this project is the optimization of physical and nutritional parameters of bacteriocins produced by probiotic bacteria. These results indicate that bacteriocin acts bacteriocidally rather than bacteriostatic ally on sensitive cells.

The nature of carbon sources also directly influences the growth and bacteriocin yield of probiotic strain. The maximum culture density was found to be observed with starch. However, growth of bacteria with sucrose and lactose was found almost similar. Todorov *et al.*, (2004, 2005) proved that, a Latic acid bacteria a strain isolated from goat milk, has been shown to increase the yield of bacteriocin with up to 2% supplement of starch.

Nitrogen source is another important nutritional supplement required for growth and bacteriocin production. Each evaluated nitrogenous source has shown a differential effect on growth, however, each nitrogen source has supported the growth and the bacteriocins production. The meat extract has shown the maximum cell mass among the tested nitrogen components. The tryptone and peptone provided second best factor contributing growth and bacteriocin yield, which is followed by yeast extract.

These influence of nitrogen sources on bacteriocin activity, showed a linear relationship with cell mass and bacteriocin production. A variable effect of meat extract, tryptone and peptone on growth and

bacteriocin production of many *Bacilli* has been evaluated (Nel *et al.*, 2001; Lee *et al.*, 2012). A high level of bacteriocin production has been observed with MRS broth supplemented with peptone while reducing levels of biomass with this combination was observed (Lee *et al.*, 2012).

In this experiment, the maximum growth of isolated cell was recorded with 1% NaCl concentration. The bacteriocin inhibitory activity was also found to be optimum at 1% NaCl salt concentration. However, the least cell biomass and bacteriocin production were observed at 2% and 4% NaCl concentration $OD_{540 \text{ and } 620}$ nm. This experimental feature on cell growth and bacteriocin activity, further support the growth associated bacteriocin production as it was observed with other performed culture parameter. The best growth of isolated strain was found to be maximum at the 1% and 3% NaCl concentration. The optimum bacteriocin activity with 1- 3 % NaCl concentration has been previously reported (Palanisamy *et al.*, 2013).

Among the conducted set of pH experiments, the optimum inhibitory of probiotic strain was observed in the range of pH 6 – 7. The maximum amount of bacteriocin was found to be produced nearly at pH 6. However, further increased in pH 7, the bacteriocin production was found to be militated against *Vibrio parahaemolytics*. The least biomass and bacteriocin yield were observed at pH 5 and pH 8. These finding suggested that bacteriocin production increased near the optimum condition of temperature and pH, but highly specific to producer strain.

The effect of temperature on cell growth and bacteriocin production from probiotic bacteria was found to be observed from 15- 40ºC and it was optimized at 30^0C. The bacteriocin activity was found to be reduced after 15ºC. These Result indicated that optimum level of bacteriocin was associated with an optimum temperature of growth. The growth associated optimum bacteriocin production at 30ºC from *Vibrio parahaemolytics, Vibrio cholera* has been reported (Palanisamy *et al.*, 2013; Juarez *et al.*, 2002).

Yang *et al.*, (2012) reported that the lactic acid bacteria were thermally stable at 60^0 C, 70^0C, 80°C and 90°C for 10 minutes. In this study, the activity of these compounds were significantly maintained their activity by boiling for 10 minutes, which indicates that the antimicrobial activity of the culture supernatant is heat-resistant to *v. cholera* and sensitive to *V. parahaemolytics* and *L. monocytogens*.

The study concluded that the isolated probiotic C2 strain from human milk meet several of the probiotic criteria, which includes acid and bile tolerances, as well as the production of antimicrobial substances. These characteristics may be advantageous for a probiotic culture to be successful in colonizing and compete with pathogens in gastrointestinal environment. The ability of these isolates to survive in acidic conditions, bile resistance, and the production of bacteriocin that is active against enteric pathogens and useful in prevention of enteric infections. These bacteriocins were also stable over a wide range of pH, temperature and heat. This heat, temperature and pH stability may be useful if the bacteriocin is to be used as an antimicrobial agent in fermented foods or thermally processed foods. Thus, these probiotic strains could be used for both preventive and therapeutic purpose in controlling intestinal infection

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