Journal of Genetic Engineering and Biotechnology (2016) **14**, 91–97

HOSTED BY

ELSEVIER

Academy of Scientific Research & Technology and National Research Center, Egypt

Journal of Genetic Engineering and Biotechnology

www.elsevier.com/locate/jgeb

ORIGINAL ARTICLE

In vitro study of probiotic properties of *Lactobacillus plantarum* F22 isolated from chhang – A traditional fermented beverage of Himachal Pradesh, India

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Received 19 July 2016; accepted 14 August 2016
Available online 29 August 2016

KEYWORDS

Himachal Pradesh;
Chhang;
Lactic acid bacteria;
Lactobacillus plantarum;
Probiotic

Abstract Present study was carried out to evaluate a new bacterial strain, *Lactobacillus plantarum* F22 as probiotic strain. *L. plantarum* F22 was isolated from a traditional inoculum called 'Phab' which is used for the preparation of a traditional beverage chhang of Lahaul and Spiti of Himachal Pradesh. The isolate was identified by conventional and molecular techniques and tested for different probiotic properties. The 16S rRNA sequence of the isolate was registered in National Centre for Biotechnology Information (NCBI) under accession number **KT865223**. Further, *L. plantarum* F22 was evaluated for its probiotic potential viz., autoaggregation capacity, hydrophobicity, acidity tolerance, antibiotic susceptibility and cumulative probiotic potential and was found to possess good probiotic potential with a cumulative probiotic score of 91.7%. *L. plantarum* F22 has been proved to be highly effective, therefore can be recommended for the development of new pharmaceuticals and functional food preparations.

1. Introduction

Himachal Pradesh is well known for its fermented food products. Being a hilly state and having a cold weather for most of the year, probably renders this pre-digested food easier for digestion besides providing high nutritional value to the hard working people of Himachal Pradesh. These traditional/indigenous foods are prepared according to the traditional methods using simple equipment's and under natural conditions from the staple material and other ingredients [32]. LAB's are common microorganisms present in fermented foods and also constitute the natural intestinal microbiota of humans and most animals [31]. LAB produces a wide range of antimicrobial metabolites which include organic acids, diacetyl, hydrogen peroxide, antibiotics and bacteriocins. Nowadays, food is no longer considered by consumers only in terms of taste and immediate nutritional needs, but also in

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Peer review under responsibility of National Research Center, Egypt.

http://dx.doi.org/10.1016/j.jgeb.2016.08.001

terms of their ability to provide specific health benefits beyond their basic nutritional value.

Probiotics are the major discussion topic of health today. Due to its increasing biomedical benefits, these were widely used by various industries for the formation of nutraceutical products. Probiotics are described as 'live microorganisms which, when administered in adequate numbers, confer a health benefit on the host [12]. Probiotics are beneficial bacteria in that they favourably alter the intestinal microflora balance, inhibition of undesirable bacteria [11], promote good digestion, boost immune function and increase resistance to infection [21]. Other physiological benefits of probiotics include removal of carcinogens, lowering of cholesterol, immunostimulating and allergy lowering effect, synthesis and enhancing the bioavailability of nutrients, alleviation of lactose intolerance [28], reduction of cholesterol levels, control of diarrhoea [13], alleviation of lactose intolerance [19], inflammatory bowel diseases [26]. They are also a source of vitamins, especially of the B group [7].

Chhang is a traditional fermented beverage produced from the spontaneous fermentation of rice by adding a traditional inoculum called 'Phab'. It is consumed by the tribal folks of Lahaul and Spiti district of Himachal Pradesh. Since, traditional fermented beverages are least explored, and so are rich repositories of rare/novel probiotic strains with immense potential of various health beneficiaries, the present study was carried out to investigate the probiotics potential of the isolated lactic acid bacteria from chhang-a famous traditional fermented beverage of Himachal Pradesh.

2. Material and methods

2.1. Isolation screening and identification of Lactic acid bacteria

Lactic acid bacterial strain was isolated from Chhang using the serial dilution and spread plate method on sterilized petriplates containing solidified media Man, Rogosa, Sharpe (MRS) at 37 °C for 48 h under anaerobic conditions [1]. Fig. 1 shows the traditional starter (Phag) culture used for isolation of Lactic acid bacteria. Anaerobic conditions were maintained under anaerobic gas jars using gas pack system (Hi-media, Make). Screening of isolates was carried out using morphological and biochemical methods and antagonistic potential [17,2]. Colour, form, margin, elevation and texture of each isolated strain were noted down. Gram's staining, catalase test, oxidase test, citrate utilization test, gas production from glucose, casein hydrolysis and H_2S production and sugar fermentation were performed with isolated strains by standard microbiological techniques [1]. The identification of the isolates was performed according to the criteria of Bergey's Mannual of Determinative Bacteriology (7thEdn.) [5]. Serious food borne/spoilage causing bacteria viz., *Staphylococcus aureus* IGMC, *Enterococcus faecalis* MTCC 2729, *Listeria monocytogens* MTCC 839, *Clostridium perfringens* MTCC 1739, *Leuconostoc mesenteroids* MTCC 107 and *Bacillus cereus* CRI were used to study

Figure 1 Phab: Traditional Starter culture used for preparation of Chhang.

antagonistic potential. The test strains were procured from Institute of Microbial Technology (IMTECH, Chandigarh, India), Central Research Institute (CRI, Kasauli, H.P. India) and Indira Gandhi Medical College (IGMC, Shimla, H.P. India). All these test strains revived twice for 24 h at 37 °C before performing experiments, as all these indicators were preserved in 40% glycerol at −20 °C. Antagonistic activity of isolates was studied by the Bit/Disc method [2,14]. Finally, bacterial strain F22 was selected on the basis of its strongest antagonistic potential for a further probiotic study. The sequence analysis of 16S ribosomal RNA gene technique (16S rRNA) was employed for identification of isolate F22. Then the sequence homologies were analysed by comparative studies using "The National Centre for Biotechnology Information (NCBI) and Basic Alignment Search Tool (BLAST). *Lactobacillus plantarum* F22 registered under the accession number **KT865223**.

2.2. Probiotic attributes

Probiotic potential of *L. plantarum* F22 was studied by evaluating various factors viz., lactic acid production, auto aggregation, acid tolerance, bile tolerance, bacteriocin production, adhesion to solvents and antibiotic sensitivity.

2.2.1. Lactic acid production

Inoculum preparation: active culture of *L. plantarum* F22 (24 h old) was inoculated (1% v/v) into 10% sterile reconstituted skim milk and incubated at 37 °C for 73 h. Samples were withdrawn every 24, 48, 72 h interval of incubation period. The pH of cultured reconstituted skim milk was measured using pH metre. The acidity was determined by titrating cultured reconstituted skim milk against 0.1 N NaOH as given below:

Acidity in terms of lactic acid: an aliquot of the sample prepared was diluted with recently boiled distilled water. 2–3 drops of 1 % phenolphthalein solution was used as an indicator and titration was done with 0.1 N NaOH. Titre value was noted and calculations were done as percent anhydrous lactic acid [29].

$$\text{Titratable acidity } (\%) = \frac{\text{Titre} \times \text{Normality of alkali} \times \text{volume made up} \times \text{equivalent weight}}{\text{volume of sample taken} \times \text{volume of aliquot taken} \times 1000} \times 100$$

2.2.2. Acid tolerance

The tolerance of the *L. plantarum* F22 to simulated gastric juices was tested [18]. The isolate was grown on MRS broth and incubated for 24 h at 37 °C. The cells were harvested by centrifugation at 5000 rpm for 10 min at 4 °C, washed twice in sterile phosphate buffer saline PBS, the cells were resuspended in PBS by lowering pH to 1, 2 and 3 followed by incubation at 37 °C in each set of pH for 30, 60 and 90 min. Total viable count was determined before and after incubation period at pH 1, 2 and 3, under aerobic conditions. Control samples without acidification were also prepared. The percent survival of cells was calculated using formula as given below:

$$\% \text{ Survival} = (\log \text{ cfu 3rd } h / \log \text{ cfu 0th } h) \times 100$$

2.2.3. Bile salt tolerance

The resistance of the strains to bile was performed according to Dora and Glenn [9]. *L. plantarum* F22 cells were inoculated into sterilized 10 ml of MRS broth containing 0.3%, 1% and 2% Ox-bile and incubated at 37 °C for 72 h. The optical density (OD) at 620 nm was measured and compared to a control culture. The percent survival of cells was calculated using formula as given below:

$$\text{Survival } (\%) = (\Delta\text{OD0\% BS} - \Delta\text{OD0.3, 1 or 2\% BS}/\Delta\text{OD0\% BS}) \times 100$$

2.2.4. Autoaggregation properties

The autoaggregation capacity of *L. plantarum* F22 was determined according to Kos et al. [25]. The culture was grown in MRS Broth for 18 h at 37 °C. The pellet was washed twice in phosphate-buffered saline (PBS) and re-suspended in similar solution to reach 10^8 cfu/ml numbers of cells. Autoaggregation was determined by measuring their absorbance at 0 h ($A0$) and after 5 h (At) and calculated using the following formula:

$$\text{Autoaggregation } (\%) = [1 - (At/A0)] \times 100.$$

2.2.5. Cell surface hydrophobicity

Bacterial adhesion to hydrocarbons was determined by the method of Mishra and Prasad [27]. *L. plantarum* F22 was harvested after growing for 18 h at 37 °C followed by centrifugation for 15 min at 5000 rpm. The cells were washed twice in Phosphate Urea Magnesium Sulphate (PUM) buffer and suspended separately in the same buffer at the level of 10^8 cfu/ml. The absorbance of the suspension was measured at 600 nm (A). Five ml of cell suspension was mixed with 1 ml of different hydrocarbon viz. xylene, toluene, ethyl acetate and chloroform. The mixture was vortexed for 1 min and the phases were allowed to separate for 1 h at 37 °C. The lower aqueous phase was carefully removed with a sterile Pasteur pipette and final absorbance (A0) was recorded at 600 nm to calculate cell hydrophobicity:

$$\text{Hydrophobicity } \% = [(A - A0)/A] \times 100$$

2.2.6. Antibiotic sensitivity test

Antibiotic sensitivity of isolated strain was determined on solid MRS medium by the use of 10 different discs of antibiotics (HiMedia®, India) and sensitivity was measured in term of zone of inhibition (Table 1).

Table 1 Antibiotics used and their concentrations.

S. No.	Antibiotic Used	Concentration (μg)
1.	Ampicillin	10
2.	Gentamicin	10
3.	Nalidixic	30
4.	Chlorophenicol	30
5.	Cifrofloxacin	5
6.	Tetracycline	30
7.	Amoxyclove	30
8.	Co-trimoxazol	25
9.	Vancomycin	30
10.	Methicillin	30

2.2.7. Bacteriocin production during growth phase

100 ml of MRS broth (pH 6.5 ± 2) was seeded with active bacterial isolate *L. plantarum* F22 @ 10% (1.0 OD). Bacterial isolate was incubated in orbital shaker at 35 ± 2 °C with a shaking speed of 120 rpm for 90 h. OD_{520} and bacteriocin production of isolate was detected periodically after every 2 h. To detect bacteriocin production, the culture of *L. plantarum* F22 was centrifuged after every 2 h at 18,000 rpm at 4 °C for 20 min. The supernatant was filtered and collected in a sterilized test tube. Well diffusion method was repeated with this preparation against indicators *E. faecalis* MTCC 2729, *S. aureus and L. monocytogens*. Zone of inhibition was recorded after every 2 h of collected supernatant. The bacteriocin production was examined and exact time of bacteriocin production was noted down.

2.2.8. Bacteriocin production

100 ml of MRS broth (pH 6.5 ± 2) was seeded with active bacterial isolate *L. plantarum* F22 @ 10% (1.0 OD). Bacterial isolate was incubated in orbital shaker at 35 ± 2 °C with a shaking speed of 120 rpm for 36 h. This collected supernatant was neutralized to pH 7.0 (with sterilized 1 N NaOH) and catalase was added (2 mg in 20 ml). Further bacteriocin activity in cell free supernatant was determined by activity unit per milliliter (AU/ml) [24,15].

2.2.9. Effect of enzymes – pepsin, trypsin, proteinase k and amylase on the activity of bacteriocin

Effects of proteolytic enzymes on bacteriocin production by *L. plantarum* F22 was checked after neutralizing the effect of acids and H_2O_2 with 1 N NaOH and Catalase. 0.25 mg of each proteolytic enzyme viz. pepsin, trypsin, proteinase K and amylase was dissolved in 1 ml of 0.5 M phosphate buffer and then added to supernatant in the ratio of 1:1. Supernatant after neutralizing the effect of acids and H_2O_2 with 1 N NaOH and Catalase was taken as control. The preparations C, ER1, ER2, ER3 and ER4 were incubated for 1 h at 37 °C. The enzyme reaction and both the enzyme control were assayed by well diffusion method of Kimura et al. [24].

3. Results and discussion

3.1. Antagonistic potential

Antagonistic potential of *L. plantarum* F22 was tested against selected food borne/spoilage causing bacteria viz., *S. aureus* IGMC, *E. faecalis* MTCC 2729, *L. monocytogens* MTCC 839, *C. perfringens* MTCC 1739, *L. mesenteroids* MTCC 107 and *B. cereus* CRI. The data on inhibitory spectrum of the isolate by bit/disc method is shown in Table 2. Among all isolates, *L. plantarum* F22 showed broadest and strongest antagonism ranging from (12 to 25 mm) against all the test indicators, thus selected for further studies. The wide spectrum inhibitory activity against challenging food borne pathogens make this isolate desirable for exploring their potential for health benefits in production of functional food. Similar studies were reported by Gautam and Sharma [16], where *Lactobacillus spicheri* G2 showed 60% of antagonism against various test indicators tested by them.

3.2. Probiotic attributes

Probiotic attributes of *L. plantarum* F22 were studied viz., lactic acid production, acidity tolerance, bile tolerance, autoaggregation, hydrophobicity and antibiotic sensitivity.

3.2.1. Lactic acid production

Lactic acid production by the LAB's is one of the important criteria for its use as probiotic strain as this acid is a secondary metabolite which often plays important role in defence mechanism by inhibiting the pathogenic bacteria and thus aids in colonization of LAB [16]. Maximum lactic acid is produced during stationary phase. The lactic acid was measured by the standard method as described by Ranganna [29]. Production of lactic acid during growth phase of *L. plantarum* F22 has been presented in Table 3. At 0 h, lactic acid production by *L. plantarum* F22 was minimum i.e., 0.24% while pH was maximum at 6.5. It changes to 0.71% with pH 4.42, 1.05% pH 4.21% and 1.03% with pH 4.12 after 24, 48 and 72 h of growth. Statistically, correlation studies revealed that there was a negative relationship between lactic acid concentration and pH during the growth phase ($r = -0.945$ for *L. plantarum* F22) i.e., lactic acid production is minimum when pH is maximum and vice versa. Present study is in accordance to early reports where authors have claimed the negative correlation between lactic acid concentration and pH [15].

Table 3 Estimation of Lactic acid produced by *L. plantarum* F22.

Time duration (h)	Lactic acid (%)	pH
0	0.24	6.50
24	0.71	4.42
48	1.05	4.21
72	1.03	4.12

Table 4 Potential of *L. plantarum* F22 for acid tolerance.

pH	Cell survival (Log cfu/ml)* Incubation time (min)					**% cell survival Incubation time (min)		
	0	60	120	180	Mean	60	120	180
1.0	6.008	5.888	5.612	4.794	5.575	98.0	93.4	79.7
2.0	6.042	6.034	5.982	5.611	6.001	99.9	99.0	92.8
3.0	6.059	6.066	6.034	5.982	6.035	100	99.6	98.7
Control	6.082	6.081	6.083	6.081	6.082	100	100	100

* Log cfu/ml: mean of results from three separate experiments.
** % survivability = (log cfu 3rd h/log cfu 0th h) × 100.

3.2.2. Acid tolerance

Before reaching the intestinal tract, probiotic bacteria have to survive in the transit through the stomach where pH can be as low as 1.5–2 [10]. Therefore acid tolerance of *L. plantarum* F22 was tested by suspending bacterial cells in phosphate buffer saline of different pH 1.0, 2.0 and 3.0 following incubation for 60, 120 and 180 min. *L. plantarum* F22 showed remarkable survival of 90.4% after 180 min at pH 1.0, whereas, at pH 2.0 and 3.0 it showed survival of 97.2% and 99.4% at pH 2.0 and 3.0 as long as 180 min of incubation period as shown in Table 4. A similar study was reported by Gautam and Sharma, [15], who studied the effects of low pH (1, 2 and 3) on the viability of the *Lactobacillus brevis* UN. *L. brevis* UN showed 91.87% of survival at pH 1.0 after 3 h, whereas, it showed 100% survival by the control after 3h.

3.2.3. Bile salt tolerance

Tolerance to bile salts is considered to be a prerequisite for colonization and metabolic activity of bacteria in the small intestine of the host [20]. Therefore, when evaluating the potential of using microorganisms as effective probiotics it is generally considered necessary to evaluate their ability to resist the effects of bile acids. *L. plantarum* F22 was found to tolerate 0.3% of bile concentration. Our results were in accordance with one of the studies where, Boke et al. [4] had shown bile

Table 2 Antagonistic spectrum of *L. plantarum* F22 by Bit disc/well diffusion method in terms of zone size.

Methods	*S. aureus*	*E. faecalis*	*L. monocytogens*	*C. perfringens*	*L. mesenteroids*	*B. cereus*	% inhibition
Bit disc method	23.0	19.0	19.0	20.0	23.0	17.0	100
Well Diffusion method	22.0	30.0	28.0	23.5	20.0	17.0	100

Antagonistic activity in terms of inhibitory zone (mm).
$*\text{Percent Inhibition}(\%) = \frac{\text{No.of inhibited indicators}}{\text{Total No.of Indicators}} \times 100$

salt tolerance at 0.3 % bile concentration of two strains of *L. delbrueckii* subsp. *bulgaricus*. *L. delbrueckii* subsp. *bulgaricus* (B3 and G12) strains showed survival of 36% and 33%, respectively.

3.2.4. Autoaggregation on the basis of sedimentation rate

The sedimentation rate of *L. plantarum* F22 was measured over a period of 5 h. Initially, the percentage of autoaggregation was 31.4 and in the final 5th h, the autoaggregation registered a high percentage of 79.5% proving isolate as strong autoaggregating phenotype. The observed autoaggregation had been related due to cell surface component, because it was not lost after washing and suspending of the cells in PBS (Fig. 2).

3.2.5. Bacterial adhesion to solvents

The ability of probiotics to adhere to epithelia is studied *in vitro* by evaluating the cells surface hydrophobicity towards xylene, toluene, chloroform and ethyl acetate. *L. plantarum* F22 showed 50.8% adhesion towards xylene. The isolate was found to be highly hydrophobic (Table 5).

The adherence to gut is an important criterion to select probiotic bacteria as therapeutic agents. Indeed, the probiotic ability to adhere to the intestinal epithelium is regarded as a prerequisite to colonize the human GIT for exerting beneficial effects, such as the exclusion of enteropathogenic bacteria [6,23]. Autoaggregation of probiotic strains appears to be necessary for adhesion to epithelial cells, with coaggregation resulting in a barrier that prevents colonization by pathogenic microorganisms [30,8]. Similar studies were reported by Kos et al. [25], where strain *Lactobacillus acidophilus* M92 exhibited a high degree of hydrophobicity determined by microbial adhesion to xylene i.e., 70.96%.

3.2.6. Sensitivity to antibiotics

L. Plantarum 22 was found to be sensitive to majority of tested antibiotics viz., Ampicillin (10 µg), Gentamycin (10 µg), Nalidixic (30 µg), Amoxyclove (30 µg), Chloromphenicol (30 µg), Tetracycline (30 µg), Cifrofloxacin (5 µg), Co-trimoxazol (25 µg), Methicillin (30 µg), and Vancomycin (30 µg), etc. The antibiotic susceptibility of strain is crucial from the safety point of view for their use as potential probiotics because

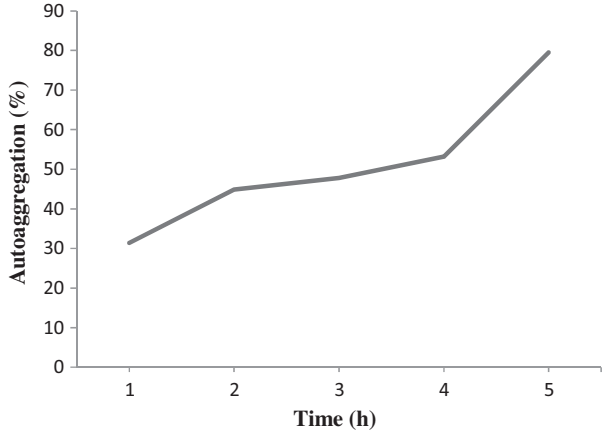

Figure 2 Autoaggregation ability of *L. plantarum* F22.

Table 5 Adhesion of *L. plantarum* F22 to different hydrocarbons.

S. No.	Name of hydrocarbon	OD_{600}*	% Hydrophobicity**
1.	Xylene	0.568	50.8
2.	Toluene	0.715	36.1
3.	Chloroform	0.734	9.0
4.	Ethyl Acetate	0.594	20.4

* OD: Mean (±Standard Deviation) of results from three separate experiments.
** Hydrophobicity %: AO-(At/AO).

probiotic bacteria may act as potential reservoir of antimicrobial resistance genes and which can be transferred to gastrointestinal tract. Similarly, Hoque et al. *[22]*, tested susceptibility and resistance against various antibiotics. The results showed that, their isolate *Lactobacillus* spp. isolated from Bogra yoghurt were sensitive to amoxicillin, moderately sensitive to gentamycin, clindamycin, azithromicin and resistant to kanamycin, nalidixic acid, metronidazol, cefradine and tetracycline, whereas, *Lactobacillus* spp. isolated from yoghurt of Khulna region were sensitive to gentamicin, clindamycin and resistant to amoxicillin, tetracyclin, kanamycin, nalidixic acid, metronidazol, azithromicin and cefradine.

3.2.7. Inhibitory spectrum of L. plantarum F22 during their growth phase

The growth curve of the isolates followed a sigmoid curve pattern based on measuring bacterial turbidity level OD_{540} nm. The bacterial cultures were incubated at 37 °C in MRS broth (6.5 pH) for different interval of time (6–90 h). Optical density and inhibition zones were measured after 6 h of interval at 540 nm. The growth was initiated at 0 h with optical density of 0.065 in *L. plantarum* F22. The log phase has been extended between 24 and 42 h and stationary phase prevailed between 42 and 78 h. The maximum inhibition against 3 test pathogens taken in the present study (*E. faecalis*, *S. aureus* and *L. monocytogens*) was noticed in the late log phase and in beginning of the stationary phase. This indicated peak period of inhibition was in between 36 and 42 h (OD 1.74 onwards).

The bacteriocin production of *L. plantarum* F22 was measured on the lawns of indicators i.e. *E. faecalis* (MTCC 2729) after neutralizing the effect of acids and H_2O_2 produced by them Fig. 3(A). The inhibitory activity revealed the presence of bacteriocin produced by the strain. Bacteriocin production was estimated in terms of activity units of culture supernatant. The activity units were found to be 2×10^3 AU/ml.

Bacteriocins are proteinaceous compounds or carbohydrate moieties which contribute significantly to inhibit the growth of pathogenic microorganism other than primary metabolites of the isolates. The inhibitory action of LAB is mainly due to accumulation of main primary metabolites such as lactic and acetic acids, ethanol, carbon dioxide; or antimicrobial compounds such as formic, benzoic and acids, hydrogen peroxide, diacetyl and acetoin [33]. In addition, LAB has shown to possess inhibitory activities due to the bactericidal effect of protease sensitive bacteriocins. By producing these antimicrobial compounds, probiotic microorganisms gain an edge over other microorganisms to survive in the adverse conditions of gastrointestinal tract. Similarly, Gautam et al. [17], isolated a bacteriocin producing strain *Lactobacillus brevis* UN from

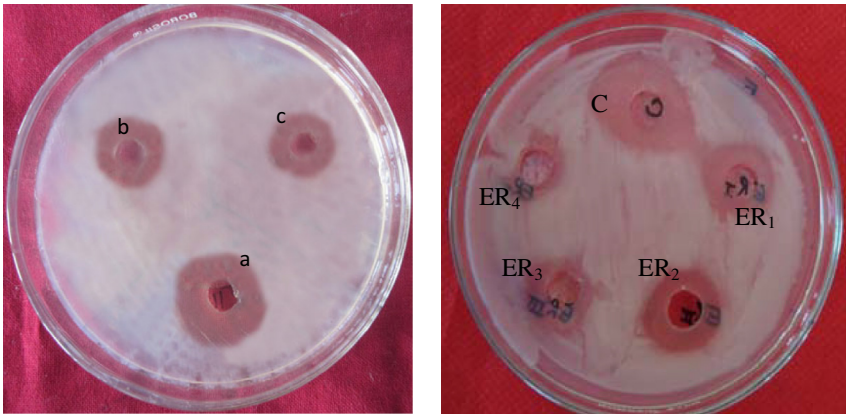

Figure 3 (A) Zone of Inhibition by *L. plantarum* F22 (a) supernatant (b) supernatant with neutral pH (c) catalase treated supernatant; (B) effect of different enzymes on bacteriocin produced by *L. plantarum* F22.

Dulliachar and the strain was found to produce bacteriocin with broad spectrum activity against spoilage causing/food borne pathogens. The maximum bacteriocin production was shown at early stationary phase.

3.2.8. Effects of Enzymes on bacteriocin

The effect of amylolytic and proteolytic enzymes was studied on *L. plantarum* F22 bacteriocin. The bacteriocin of *L. plantarum* F22 was treated with proteolytic enzymes a decrease was observed in the zone size after the treatment. The results revealed that there was 53.2–67.2% decrease in the zone size with proteolytic enzymes and 13.2% decrease was observed with amylase Fig. 3(B).

To confirm the proteinaceous nature of bacteriocin, the supernatant has been treated with various enzymes, and this study has also been well documented in literature where, Bhattacharya and Dass [3] observed that antimicrobial compounds produced by the isolates were inactivated by all the proteolytic enzymes (pepsin and trypsin) whereas, no reduction in the zone was encountered when the bacteriocins were treated with amylase, catalase and lipase.

4. Conclusions

Results obtained in this study showed that this probiotic strain *L. plantarum* F22 isolated from chhang was capable of tolerating high bile salt, able to survive in simulated low gastric pH and showed broadest antagonism against a wide range of food borne/spoilage causing bacteria. In addition, the isolate was found sensitive to most of the antibiotics used, had strong autoaggregation and hydrophobicity. Therefore, *L. plantarum* F22 has been proved to be highly effective, therefore can be recommended for the development of new pharmaceuticals and functional food preparations for public health.

Acknowledgement

The financial support received from the Department of Biotechnology, Govt. of India, New Delhi, India to carry out this piece of work is highly acknowledged.

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