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Effect of medium components on bacteriocin production by *Lactobacillus plantarum* LABF1, isolated from fish

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Abstract: **The cell-free supernatant containing bacteriocin LABF1, produced by *Lactobacillus plantarum* LABF1, inhibits the growth of food borne bacterial pathogens viz., Listeria monocytogenes MTCC 657, Bacillus cereus MTCC 1272, Staphylococcus aureus MTCC 740, Salmonella typhi MTCC 531 and Escherichia coli MTCC 2939. Strain *Lactobacillus plantarum* LABF1 produces bacteriocin LABF1. A maximum total bacteriocin activity of 6400 AU/ml was recorded after 14 h in MRS broth. In MRS broth adjusted to pH=5.5, 6.0 or 6.5, an equal level of bacteriocin production of 6400 AU/ml was recorded. Optimal production (6400 AU/ml) was recorded in the presence of tryptone (20 g/L), a combination of tryptone and meat extract (12.5+7.5 g/L), or tryptone and yeast extract (12.5+7.5 g/L). In the presence of 20 g/L of sucrose and maltose yielded the activity levels of 6400 AU/ml. Increasing the Concentrations of 5.0 to 20g/L K2HPO4 or 2 to20g/L of KH2 PO4 had a negative effect on bacteriocin production. Concentration of 2.0 ml/L of tween80 yielded double activity (12800 AU/ml). Supplementing MRS with 1 ml/L or more Concentrations of glycerol reduced the production of bacteriocin. Growth in the presence of vitamins did not stimulate bacteriocin production.**

Keywords: **Bacteriocin, *Lactobacillus plantarum*, fish.**

1. INTRODUCTION

Lactic acid bacteria are widely used as starter cultures and play an important role in food preservation, microbiological stability and production of aroma compounds(Deegan *et al.*,1992, klaenhammer *et al.*,1993, Requena *et al.*, 1995) . Many of these lactic acid bacteria produce bacteriocins (klaenhammer *et al.*, 1993, Requena *et al.*, 1995). By definition, bacteriocins are small proteins with bactericidal or bacteriostatic activity against genetically closely related species (klaenhammer *et al.*, 1993).

They are generally low molecular weight proteins that gain entry into target cells by binding to cell surface receptors. Their bactericidal mechanism varies and may include pore formation, degradation of cellular DNA, disruption through specific cleavage of 16S rDNA, and inhibition of peptidoglycan synthesis(De Vuyst and Vandamme, 1994) . Lactic acid bacteria are gram positive, non-sporulating bacteria hold a prominent place in the production of bacteriocins. Phylogenetically, lactic acid bacteria belong to the gram positive *Clostridium-Bacillus* sub-class. Traditionally, the lactic acid bacteria have been used as starter cultures for the fermentation of various foods and beverages since they contribute to flavour and aroma and retard spoilage (Hechard and Sahl, 2002) . Lactic acid bacteria are non-pathogenic grows rapidly, requires very cheap substrates. Hence, lactic acid bacteria were used as industrial microorganisms for the synthesis of various chemicals, pharmaceuticals, and other products useful to humans (Jack *et al.*, 1995) . In recent years, the researchers paid much attention to isolate large variety of bacteriocins producing lactic acid bacteria from different sources, for their applications as food bio-preservatives, since they can be degraded only by gastrointestinal protease (Jamuna and Jeevaratnam,2004) . Bacteriocinogenic lactic acid bacteria and / or their isolated bacteriocins are considered as safe additives useful to control the frequent development of pathogens and spoiling microorganisms in foods and feed.

The spreading antibiotic resistance and the demand for products with fewer chemicals create the necessity of exploring new alternatives in order to reduce abusive use of therapeutic antibiotics. In this context, bacteriocins are indicated to prevent the growth of undesirable bacteria in a food grade and more natural way, which is convenient for health and accepted by the community (Parada *et al.*, 2007). The studies on the same require the production of the bacteriocin in large amounts for which conditions should be optimized for the maximum production of bacteriocin by the bacterium. In this paper we report the production of bacteriocin LABF1and from *L. plantarum*, a strain isolated from fish. The effects on the nutrients and medium pH on bacteriocin activity was determined.

2. MATERIALS AND METHOD

Bacterial strain and growth media

Bacterial Strain LABF1was isolated from fish and identified as *L. Plantarum* on the basis of growth on selective MRS agar, cell morphology, gram staining, catalase activity and biochemical identification of LABF1. Further identification of the species of these LABF1 was performed according to carbohydrate fermentation patterns and growth on MRS broth (HI Media) as described in Bergey's manual of systematic bacteriology.

The isolated LABF1 were sub cultured and the purified cultures maintained at MRS agar slants. MRS medium (HI Media) was used for all experiments, except in growth optimization in which case modified MRS broth(De Man *et al.*, 1960) was used.

Bacteriocin bioassay

Bacteriocin screening was performed by using the well diffusion method (Ivanova *et al.*, 1998). Adjustment of the cell-free supernatant to pH 6.0 with 1M NaOH prevented the inhibitory effect of lactic acid. Antimicrobial activity was expressed as arbitrary units (AU) per ml. One AU was defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition(Ivanova *et al.*, 1998*).* The indicator strain was listed in table - 1.

Production of bacteriocin LABF1 in different initial growth pH

The effect of initial medium pH on the production of bacteriocins, LABF1 was determined. Volumes of 300 ml MRS broth were adjusted to pH 4.5, 5.0, 5.5, 6.0 and 6.5, with 6M HCl or 6M NaOH and then autoclaved. Each flask was inoculated with 2% (v/v) of an 18-h-old culture of strain LABF1 respectively, and incubated at 30ºC for 20 h without agitation. Changes in culture pH and production of bacteriocin LABF1, expressed as AU/ml, were determined every hour as described as earlier. All experiments were done in triplicate.

Effect of medium composition on the bacteriocin LABF1 production.

Strains LABF1 was grown in 10 ml MRS broth for 18 h at 30ºC, the cells harvested by centrifugation (8000g, 10 min, 41ºC), and the pellet resuspended in 10 ml sterile peptone water. Four millilitres of this cell suspension was used to inoculate 200 ml of the following media: (a) MRS broth (Hi media), i.e. control with 20.0 g/L glucose; (b) MRS broth (De Man *et al.*, 1960) without glucose, supplemented with 20.0 g/L fructose, sucrose, lactose, maltose and mannose respectively; (c) MRS broth(De Man *et al.*, 1960), without organic nutrients, supplemented with tryptone (20.0 g/L), meat extract (20.0 g/L), yeast extract (20.0 g/L), tryptone (12.5 g/L) plus meat extract (7.5 g/L), tryptone (12.5 g/L) plus yeast extract (7.5 g/L), meat extract (10.0 g/L) plus yeast extract (10.0 g/ L), or a combination of tryptone (10.0 g/L), meat extract (5.0 g/L) and yeast extract (5.0 g/L), respectively; (d) MRS broth (De Man *et al.*, 1960) with 2.0–20.0 g/L K_2HPO_4 or 2.0–20.0 g/L KH_2PO_4; and (f) MRS broth(De Man *et al.*, 1960) supplemented with 0–20.0 ml/L glycerol. (g) MRS broth(De Man *et al.*, 1960) with 1-2ml/L tween80. In a separate experiment, the vitamins cyanocobalamin, thiamine, DL-6, 8 thioctic acid and L-ascorbic acid) were filter-sterilized and added to MRS broth at 1.0 ppm (final concentration). Incubation for all tests was at 30ºC for 20h. Activity levels of bacteriocins LABF1 was determined as described before. All experiments were done in triplicate.

3. RESULTS AND DISCUSSION

Bacteriocins of lactic acid bacteria have the potential as food bio preservatives to control pathogenic and spoilage bacteria. In this study, lactic acid bacteria were isolated from fish. Microscopic identification of the isolate could determine the rod shaped cells, gram positive, catalase negative, non-motile rods and oxidase negative which indicated the typical basic characteristics of *Lactobacilli*. Based on the carbohydrate utilization pattern of bacterial isolates were

identified as *Lactobacillus plantarum*. Similar characters for lactic acid bacteria observed earlier by Kandler and Weiss (1986). Seema Nair and Surendran (2009) in their studies isolated and characterized the lactic acid bacteria from fish and prawn. The lactic acid bacterial isolate was tested for their inhibitory activity over some food borne bacterial pathogens *Listeria monocytogenes* MTCC 657, *Bacillus cereus* MTCC 1272, *Staphylococcus aureus* MTCC 740, *Salmonella typhi* MTCC 531 and *Escherichia coli* MTCC 2939. Almost all pathogens were inhibited by the bacteriocin producer. The results are tabulated in Table-1. In MRS broth (Himedia) adjusted to pH=5.5, 6.0 or 6.5, bacteriocin LABF1 production of 6400 AU/ml was recorded (Table 2). However, at pH=4.5 low levels of bacteriocin LABF1 (1600 AU/ml) was recorded (Table - 2). Similar results were reported for other bacteriocins produced by *L. plantarum* (Jiminez – diaz *et al.*,1993 . The end-pH values of the cultures ranged between 3.7and 3.9 (Table - 2), irrespective of the initial growth pH. From these results and the literatures of (Jiminez – diaz *et al.*,1993 ,kato *et al.*,1994, Todorov *et al.*, 2000), it can be concluded that optimal production of *L. plantarum* bacteriocins occurs during early logarithmic growth, usually at a pH above 4.5. Bacteriocin LABF1 was produced at 6400AU/ml when strain LABF1 was grown in MRS broth.

The same level of activity was recorded when glucose was replaced by 20 g/L sucrose , maltose .Decrease in Bacteriocin LABF1activity was recorded in presence of 20 g/L fructose (or) lactose (or) mannose. This results suggesting that glucose moiety of sucrose is favoured the production (Table - 3). Our results showed that Bacteriocin LABF1production stimulated by glucose Similar findings were also reported by Todorov (2008) who reported that bacteriocin AMA-K produced by *L. plantarum* AMA-K, recorded the highest bacteriocin AMA-K production in MRS broth supplemented with Glucose (20.0 g/L).Of all nitrogen sources tested In supplementation of different organic nitrogen sources did not increase the bacteriocin production. The optimum bacteriocin (LBF1) production and activity was recorded in MRS broth supplemented with Tryptone (20.0 g/L), Tryptone + Yeast extract (12.5+7.5 g/L), Tryptone + Yeast extract + Meat extract (10.0 + 5.0 + 5.0 g/L) followed by MRS broth supplemented with 20.0 g/L of yeast extract and tryptone + meat extract (12.5+7.5 g/L) which recorded the bacteriocin production and activity of 3200 AU ml^{-1}. The lowest bacteriocin production and activity of 1600 AU ml^{-1} was recorded in MRS broth supplemented with Meat extract (20.0 g/L), Meat extract + Yeast extract (10.0+10.0 g/L). From these results it can be concluded that bacteriocin LABF1 production is stimulated by tryptone and not yeast extract or meat extract. Similar results were recorded for plantaricin 423 production and higher activity levels were recorded when the producer strain was grown in the presence of tryptone compared to meat extract (Verellen, *et al.*, 1998).

Little is known about the influence of potassium ions on the production of bacteriocins. Levels of 2.0 g/L KH_2PO_4 are optimal for the production of bacteriocin LABF1 (6400 AU ml^{-1}) Replacement of KH_2PO_4 with 2.0 to 20.0 g/L of K_2HPO_4 not effecting bacteriocin LABF1 production (Table - 3) .The changes in activity cannot be due to pH changes caused by higher potassium levels, since all media were adjusted to pH 6.5 before inoculation. In the presence of glycerol at $1ml^{-1}$ recorded optimum level of bacteriocin production, increasing concentration of glycerol showed reduction in bacteriocin production, these results strongly support the view expressed by Todorov *et al.*,(2000). Glycerol is not used as a carbon source and the decrease in bacteriocin production may be due to changes in osmotic stress. No difference in bacteriocin production when strain LABF1 was grown in the presence of $1.5ml^{-1}$ tween80. Concentration of 2.0 ml^{-1} tween80 yielded double activity Similar results observed by Todorov *et al*., (2012) who reported that the bacteriocin ST22Ch produced by *Lactobacillus sakei* ST22Ch recorded the highest bacteriocin production in addition of 2.0 ml L^{-1} and 3.0 ml L^{-1} of Tween80 to MRS broth. Increasing the concentrations of Tween80 in MRS broth up to 5.0 ml L^{-1} has a stimulating effect of bacteriocin ST22Ch production. Addition of vitamins to MRS broth has a negative effect on bacteriocin LABF1 production (Table - 3) Similar results were reported by bacteriocin ST 194BZ produced by *Lactobacillus plantarum* ST 194BZ(Botes *et al*.,2007); **V**on Mollendorff (2009).

4. CONCLUSION

The bacteriocin suspension of *Lactobacillus plantarum*LABF1showed the broad spectrum of inhibitory activity against food borne bacterial pathogens .The present study concluded that supplementation of MRS broth with 2.0 ml^{-1} of Tween80 increased the production of bacteriocin *Lactobacillus plantarum*LABF1.The bacteriocin LABF1or *Lactobacillus plantarum*LABF1strain have the potential for using them as bio preservatives in the food and food products.

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Table-1. Inhibitory activity of cell -free supernatant of strain LABF1 against food borne bacterial pathogens (Well diffusion method)

S. No	Indicator strain	Bacteriocin activity
1	*Listeria monocytogenes* MTCC 657	-
2	*Bacillus cereus* MTCC 1272	-
3	*Staphylococcus aureus* MTCC 740	-
4	*Salmonella typhi* MTCC 531	-
5	*Escherichia coli* MTCC 2939	-

+ , inhibition zone ; - no inhibition zone recorded

Table - 2. Influence of initial growth pH on the antimicrobial activity of bacteriocin LABF1

Initial PH	4.5	5.0	5.5	6.0	6.5
Final pH	3.7	3.7	3.8	3.9	3.9
Bacteriocin activity(AU/ml)	1600	3200	6400	6400	6400
Bacteriocin activity*	25	50	100	100	100

*Compared to the highest activity (6400 AU/ml)

Table - 3. Effect of carbohydrates, organic nitrogen, potassium, glycerol, tween80 and vitamins on bacteriocin LABF1 production

Component	Concentration (g/L)	Bacteriocin activity (AU/ml)	Percentage of activity* %
Fructose	20	1600	25
Sucrose	20	6400	100
Lactose	20	1600	25
Maltose	20	6400	100
Mannose	20	1600	25
Tryptone	20	6400	100
Meat extract	20	1600	25
Yeast extract	20	3200	50
Tryptone + Meat extract	12.5+7.5	3200	50
Tryptone + yeast extract	12.5+7.5	6400	100
Meat extract+ Yeast extract	10+10	1600	25
Tryptone +Meat extract+ Yeast extract	10.0+5.0+5.0	6400	100
K_2HPO_4	5.0	1600	25
K_2HPO_4	10.0	1600	25
K_2HPO_4	20.0	800	12.5
KH_2PO_4	2.0	3200	50
KH_2PO_4	5.0	800	12.5
KH_2PO_4	10.0	800	12.5
KH_2PO_4	20.0	0	0
Glycerol(ml)	1.0	6400	100
Glycerol(ml)	2.0	3200	50
Glycerol(ml)	5.0	800	12.5
Glycerol(ml)	10.0	800	12.5
Glycerol(ml)	20.0	0	0

Tween80(ml)	1.5	6400	100
Tween80(ml)	2.0	12800	200
Cyanocobalamin	1ppm	1600	25
Thiamine	1ppm	3200	50
DL-6,8-thiotic acid	1ppm	1600	25
L-Ascorbic acid	1ppm	3200	50
Control(MRS broth)	0	6400	100

*Compared to the highest activity (6400 AU/ml), as recorded with the control (MRS,Himedia)

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