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PURIFICATION AND CHARACTERIZATION OF BACTERIOCIN PRODUCED BY *Lactobacillus reuteri* AF182723, A STRAIN ISOLATED FROM TRADITIONALLY PREPARED CURD

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Abstract

Bacteriocins are antimicrobial peptides produced by diverse bacterial species that inhibit or kill other microorganisms. In the food industry bacteriocins have potential application as preservative. The bacteriocin obtained from fermented broth in a crude form that may contain lactic acid, acetic acid, H_2O_2 and other antimicrobial substances. Therefore, the objectives of the study were to purify the bacteriocin and to reveal the possibility of purified compound as biopreservative. Crude bacteriocin was produced by *Lactobacillus reuteri* AF182723 isolated from traditionally prepared curd in liquid fermentation system. When the crude bacteriocin was purified by sequential three step procedure involving ammonium sulfate fractional precipitation, cation exchange (SP Sepharose) chromatography and size exclusion (Sephadex G-100 column) chromatography, the specific activity (AU/mg) of the bacteriocin increased in each step of purification and it reached 6349.2AU/mg finally from 262.3 AU/mg with the purification fold of 24.206. When the purified bacteriocin was subjected to different temperature and pH treatments, it found stable over wide temperature range (45ºC - 75ºC) and pH range (3-7). When exposed to different enzymes, the purified bacteriocin showed resistant to catalase and amylase enzymes, but showed significantly higher sensitive to pepsin and proteinase K indicating that the antimicrobial substance was proteinaceous in nature. This study concluded that bacteriocin produced by *Lactobacillus reuteri* AF182723 could be used as a potential food biopreservative. Further safety studies should be done to recommend this potential bacteriocin as food biopreservative.

Keywords: Bacteriocin, Characterization, *Lactobacillus reuteri* AF182723**,** Purification

Introduction

Lactic acid bacteria (LAB) are widely used as starter culture. Antimicrobial peptides obtained from lactic acid bacteria named bacteriocin are used as biopreservatives in the food industry. Bacteriocinis are ribosomally synthesized primary metabolic peptide that exhibits inhibitory effect on food spoilage organisms[10].Use of bacteriocin obtained from LAB as biopreservative offers several advantages. They reduce the use of chemical preservatives and extension of heat treatments and not alter the food texture, flavor and aroma[3,9]. Therefore utilization of bacteriocin as biopreservative is highly advantageous. Purification and characterization of bacteriocin are important to check applicability of bacteriocin in food industry. In general bacteriocins are classified into four main classes according to their structural, genetic, and biochemical characteristics such as class I, class II,

class III and Class IV. Class I is small peptides with molecular weight < 10 kD. Class II bacteriocins are small (<10 kDa), heat-stable, non-modified peptides cationic, hydrophobic peptides. Class III is heat labile proteins and class IV is cyclicpeptides that required carbohydrate or lipid moieties for their activity[2]. Several researchers documented purification of heterogeneous nature of bacteriocins by different combination of strategies such as ammonium sulphate precipitation, ethanol precipitation, acetone precipitation, dialysis, cation exchange chromatography, RP-HPLC, ultra filtration and gel filtration[9].

Materials and Methods

Isolation and characterization of bacteriocin produced by *Lactobacillus reuteri* AF182723

Lactobacillus reuteri AF182723 was isolated from traditionally prepared curd sample by repeated streaking on MRS agar and identified by molecular studies and biochemical studies.

Production of crude bacteriocin

Production crude bacteriocin by *Lactobacillus reuteri* AF182723 was carried out by liquid fermentation. Composition of medium was maltose (20g/l), soy bean (30g/l) with substituted with 1% NaCl and mineral solution ($MnSO_4.4H_2O$-0.05g/l, $MgSO_4.7H_2O$-0.2g/l, K_2HPO_4-2g/l, $CH_3COONa.3H_2O$-5g/l, $(NH)_3C_6H_5O_7$-2g/l)at pH 5.5. *Lactobacillus reuteri* AF182723 was inoculated and initial inoculum level was maintained as 1.2×10^6cfu/ml and incubated at 30 ºC for 4 days.

Fractional purification by Ammonium sulphate

The fermented broth was centrifuged (13000 rpm, 15 min) and pH of supernatant was adjusted to 6.5. The supernatant was filtered through 0.45µm syringe filter**.** Antimicrobial peptide from crude extract was precipitated at different ammonium sulfate concentrations. Initially filter sterilized crude broth was saturated at 20% and centrifuged (13000 rpm, 15 min). The peptide precipitate was dissolved using 0.2 M sodium phosphate buffer and antibacterial activity assay was performed by using 24 hours old culture of *Enterococcus faecalis*as as the indicator organism. To find out which level of ammonium sulphate saturation leads to precipitation of bacteriocin, already 20% saturated supernatant was saturated again at 40% saturation. Saturated solution was centrifuged and dissolved by using 0.2 M sodium phosphate buffer and antibacterial activity was measured. These procedures were repeated at 60%, 80% & 100% saturation level.

Purification by cation exchange chromatography (SP Sepharose fast flow coloum)

Initially column (10.0 cm × 1.0 cm) was equilibrated with 20 mM phosphate buffer (pH 7.0). The sample (235µl of dissolved active fraction from ammonium sulphate fractional purification) was applied to column equilibrated with sodium phosphate buffer. Column was washed with 20 mM phosphate buffer and the absorbed substances were eluted from column with 20 mM sodium phosphate + 1 M NaCl (pH 7.0). The eluted fractions were collected at every five minutes interval and tested for antibacterial activity by agar well diffusion assay against *Enterococcus feacalis.* Protein concentration in each elution was determined by Bradford protein assay. The active fractions were pooled together and concentrated by 100%

ammonium sulphate. Ammonium sulphate precipitate was dissolved in sodium phosphate buffer and further purified by sephadex G-100.

Purification by size exclusion chromatography (Sephadex G-100 column)

Initially column was washed and equilibrated with 0.1M sodium phosphate buffer (pH 7.0) and 235µl of dissolved active fraction was loaded on column and allowed for 15 minutes. Elution was conducted by using 0.1M sodium phosphate buffer at pH 7.0. Eluted fractions were collected at every five minutes interval and assayed for presence of antimicrobial component by agar well diffusion assay. Protein concentration in each elution was determined by Bradford protein assay.

Determination of Protein concentration

Protein concentration of the supernatant, ammonium sulphate fraction and chromatographic elution were determined by the method of Bradford by using bovine serum albumin as the standard (Mæhre et al., 2018 and Kruger 2002)

Antimicrobial activity assay

Agar well diffusion assay was performed as antimicrobial activity assay to detect activity of bacteriocin. All ready solidified agar plate was seeded with 100µl of indicator organism (24 h old culture suspension of 6.0×108cfu/ml). Wells were prepared by using cork borer at 10mm diameter and 100 µl of crude bacteriocin/ purified bacteriocinwas added into the each wells and incubated at 37ºC for 24 hours. After 24 hours diameter of growth inhibition zone was measured by digital vernier caliper. *Enterococcus faecalis* was used as indicator organism. All the experiments were performed in triplicates. Bacteriocinactivity was expressed as arbitrary units (AU) per ml.

Effect of heat, pH and enzyme on purified bacteriocin

To determine the effect pH on bacteriocin activity, purified filter sterilized bacteriocin pH level was adjusted between 2- 10. Residual activity was treated bacteriocin solution was measured. To evaluate the effect heat on bacteriocin activity, purified filter sterilized bacteriocin was heated at 45ºC, 65ºC, 75ºC & 100ºC for 1 hour and 121º for 20 minutes. Residual activity was determined against control (purified bacteriocin without heat treatment). To evaluate the effect of enzymes, filter sterilized purified active fraction was incubated by following enzymes, Proteinase K, Catalase, Amylase at a final concentration of 0.1 mg/ml in 20 mmol/l phosphate buffer pH 7 and Pepsin at 0.1 mg /ml, 20 mmol/l of glycine HCl, pH 2. After 2 h of incubation at 37ºC, enzyme activity was stopped by heating at 100ºC for 5 min. Untreated samples were used as controls. The residual bacteriocin activity was determined by agar well diffusion assay against indicator strain *Enterococcus faecalis*.

Characterization of activity fraction

Preliminary tests in the analysis of an unknown organic compound

Purified bacteriocin was characterized. Initially preliminary tests for an unknown organic compound were carried out. Presence of alcohols, aldehydes, ketones, carboxylic acids, phenol, alkenes, esters and amines were tested.

Results and Discussion

Fractional purification by Ammonium sulphate

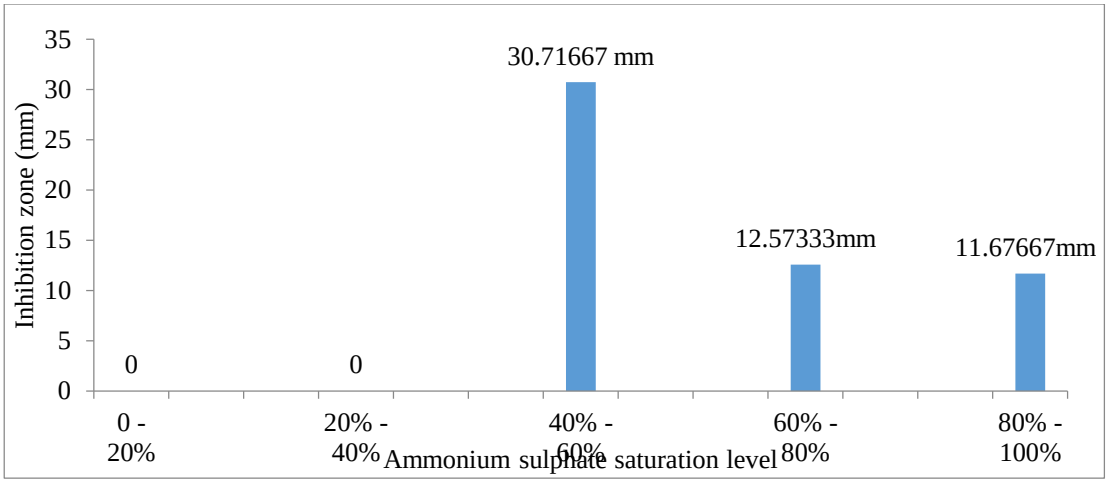

Figure 1. Inhibition zone obtained at different ammonium sulphate saturation level for *Lactobacillus reuteri* AF182723

Table 1. Antimicrobial activity of *Lactobacillus reuteri* AF182723 through purification profile

Purification steps	Volume (ml)	Activity unit (AU/ml)	Total activity (AU)	Protein (mg/ml)	Specific activity (AU/mg)	Purification fold
Culture supernatant	100 ml	320 AU/ml	32 000 AU	1.220 mg/ml	320/1.220 262.2950	1
Ammonium sulphateppt 40% - 100%	8 ml	2560 AU/ml	20480 AU	0.745 mg/ml	2560/0.745 3436.2416	13.1
SP sepharose fast flow	12 ml	160 AU/ml	1920 AU	0.029 mg/ml	160/0.029 5517.24	21.034
Sephadex G-100	24 ml	40AU/ml	960 AU	0.0063	40/0.0063 6349.206	24.206

Activity (AU/ml) = reciprocal of highest dilution with lowest observable inhibition zone (minimum observable inhibition zone = 12.00 mm), Total activity = (AU/ml) × Volume (ml), Specific activity (AU/mg) = Activity (AU/ml) / Protein concentration (mg/ml), Purification fold = Specific activity II / Specific activity I[1]

Previously bacteriocin from *Lactobacillus Viridescence* (NICM 2167) was purified in two steps involving ammonium sulphate precipitation followed by gel filtration using Sephadex G-100 increase purification fold to 4.69[8]. Purification of bacteriocin from *Lactobacillus plantarum*, by ammonium sulfate precipitation and gel filtration (Sephadex G-100 column) increase purification fold to 13.5[6]. Purification of bacteriocin from *E. faecium* M1M10 by ammonium sulphate, gel filtration, cation-exchange and hydrophobic-interaction chromatographies and reverse phase chromatography increase purification foldat 1397-fold [2]

Effect of heat, pH and heat on purified bacteriocin

Bacteriocin produced by *Lactobacillus reuteri* AF182723 was resistant to catalase and amylaseenzyme treatment. Purified bacteriocinwas found to be sensitive to pepsin and

proteinase k indicating that the antimicrobial substance was proteinaceous in nature.Purified bacteriocin was stable over wide range of pH (3-4) as well as temperature (45ºC - 75ºC).

Table 2. Effect of heat, pH and heat on purified bacteriocin from *Lactobacillus reuteri* AF182723

Treatment	Residual inhibitory activity
Catalase	40 AU/ml
Amylase	40 AU/ml
Pepsin	0
Proteinase K	0
pH 2	20 AU/ml
pH 3	40 AU/ml
pH 4	40 AU/ml
pH 5	40 AU/ml
pH 6	40 AU/ml
pH 7	40 AU/ml
pH 8	20 AU/ml
pH 9	20 AU/ml
pH 10	20 AU/ml
45ºC	40 AU/ml
65ºC	40 AU/ml
75ºC	40 AU/ml
100ºC	20 AU/ml
121º for 20 minutes	0

These results indicate that the bacteriocin produced by *Lactobacillus reuteri* AF182723could be used as biopreservative agent in acidic and thermal processed food stuff. Previously Soumya[7]*et al*., 2012 reported that supernatant produced by *Lactobacillus* was resistant to catalase treatment and found to be sensitive to temperature above 60ºC. It was stable between pH3 and 7 but sensitive to pH 9.Rattanachaikunsopon &Phumkhachorn [5]2006 stated that the bacteriocin produced by *Lactobacillus plantarum* N014 was sensitive to all proteolytic enzymes used in study, including papain, pepsin,pronase E, proteinase K, and trypsin, but was resistant to the other enzymes, such as α – amylase, lipase A, lysozyme, by 10, 20, or 30 min of boiling and autoclaved temperature. Inaddition to these findings, no change in antimicrobial activitywas detected when the culture supernatant was exposed to pH 2 to 10. However, exposing the culture supernatantto pH 1 and pH 11 to 14 eliminated antimicrobial activity.

Characterization of activity fraction

Preliminary tests in the analysis of an unknown organic compound

No changes were observed when add 2,4 DNP to test solution. Therefore acidified $K_2Cr_2O_7$ was added. Colour of acidified $K_2Cr_2O_7$(orange) was changed to green coloour.Because primary alcohols will beoxidised to aldehydes and the carboxylic acids, secondary alcohols will be oxidised to ketones.Tertiary alcohols cannot be oxidised by the dichromate ions. Dichromate ions ($Cr_2O_7^{2-}$) will be reduced Cr^{3+} ions (green). Therefore primary or secondary alcohol may be present. Again 2, 4 DNP was added, there was no

observation. It indicates that unknown purified solution may contain primary alcohol. No changes were observed when add $FeCl_3$ solution to unknown solution. But brown colour precipitate was observed after addition of $FeCl_3$ solution to already hydrolyzed unknown component by NaOH. These results indicate the presence of secondary amine in unknown purified component.

Conclusion

Purification of bacteriocin produced by *Lactobacillus reuteri* AF182723applied three steps procedure increase purification fold up to 24.206. The purified bacteriocin is highly heat stable, active at wide range of pH and degraded by gastrointestinal proteolytic enzyme. Therefore the bacteriocin from *Lactobacillus reuteri* AF182723 has highly potential applicability in food industry as biopreservative. Even though further safety studies should be done to recommend this bacterion as potential food biopreservative.

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