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Effect of *Lactobacillus acidophilus* Bacteriocin on *Bacillus cereus* Biofilms

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Lactobacillus acidophilus **produced a small bacteriocin of estimated molecular mass of 6.5 kDa. This bacteriocin exhibited strong antibiofilm activity against Gram positive food borne pathogen, *Bacillus cereus*. In addition, this antibacterial peptide also exhibited strong antagonistic activity against other Gram positive bacteria, *Staphylococcus aureus* and *Bacillus subtilis*. In sequential bacteriocin purification steps, the salt precipitated bacteriocin extract of *L. acidophilus* displayed 50.74% bacterial growth inhibition (INH%) in 25% v/v bacteriocin treatment. Mean while, the ion exchange and gel filtration chromatography extracts displayed 58.29% and 60.20% bacterial growth inhibition. Whereas in 50% v/v bacteriocin treatment, the salt precipitated, ion exchange and gel filtration chromatography extract of bacteriocin displayed bacterial growth inhibition as 72.79%, 87.12% and 96.42% respectively. The microscopic observation showed that there was an increased biofilm inhibition activity in highly purified bacteriocin extract that confirmed significant inhibition activity of bacteriocin against the formation of biofilms.**

Key words: *Lactobacillus acidophilus*, *Bacillus cereus*, Biofilm, Bacteriocin, Column chromatography.

Biofilm is an assemblage of microbial cells that is irreversibly associated to biological and non biological surfaces and is enclosed in extracellular polymeric substance matrix (Teodósio *et al*., 2011). Biofilm is a causative pollutant of food industry sectors including dairy processing, red meat processing and poultry processing. In addition, heath related fields like medicine and dentistry, and industries of oil drilling and paper production also have substantial implication of biofilm formation. Biofilm (surface associated) cells showed diverse pattern of growth as compare to planktonic (suspended) cells and are more resistant to antibacterial products. The high antibacterial resistance is due to the presence of extracellular polysaccharides which results in the formation of biofilm. These extracellular polymeric substance (EPS) provide high resistance to harsh environment, genomic change, production of secondary metabolites and modify biodegradability (Ludecke *et al*., 2014). Searching for new strategies for microbial biofilm inhibition is becoming highly necessary (Rizwan *et al.*, 2014).

The antibacterial substances including organic acids, hydrogen peroxide, reuterin and bacteriocins produced by lactic acid bacteria (LAB) were considered as generally recognized as safe (GRAS) antibacterial products for food preservations. Among them bacteriocins are extracellularly released small antimicrobial peptides and rapidly digested in human digestive tract by proteases (O'Shea *et al*., 2013). These peptides have bactericidal or bacteriostatic activity on other related and unrelated microorganisms with the

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producer having specific immunity mechanism (Dobson *et al*., 2012). Bacteriocins from LAB have been used extensively as natural preservatives by replacing synthetic compounds since 25 years and exhibit different inhibition profile on food spoilage and pathogenic bacteria (Cosentino *et al*., 2012). Recently, lots of research has been focused on enhanced production of bacteriocins against pathogenic and spoilage bacteria in vegetable foods and beverages (Collins *et al*., 2010) and their applications in pharmacy provided a good alternative to antibiotics and chemical compounds (Balciunas *et al*., 2013).

In subjected study the effect of bacteriocins produced by *Lactobacillus acidophilus* was studied for bacterial biofilm inhibition by exhibiting bactericidal effect on pathogenic bacteria which provides new natural resources to overcome the food borne biofilm related problems.

The purity of active ingredient is an important factor affecting its bactericidal activity. Thus, ammonium sulphate precipitation and column chromatography was performed to concentrate and purify bacteriocin to improve the inhibition activity against causative agent of biofilm. Many studies have been reported previously on activity of LAB bacteriocin of planktonic cells while only few citations have been reported on biofilm cells inhibition in food sector.

Methodology

Selection of culture media

The bacteriocin producing *L. acidophilus* was procured from mozzarella cheese. The culture medium used was MRS agar/broth (Oxoid, England) for *L. acidophilus* and Nutrient agar/broth (Oxoid, England) for test strains including *Bacillus cereus*. The purified colonies of *L. acidophilus* were primarily identified by catalase production and Gram staining and finally confirmed by carbohydrate fermentation profile using API 50 CHL system (Bio Merieux, France; Pringsulaka *et al*., 2012).

Bacteriocin production by *L. acidophilus*

Five hundred milliliters of MRS broth was inoculated with 1.5% (v/v) overnight seeded culture of *L. acidophilus* and incubated at 30°C for 14 h without agitation (Abo-Amer, 2011). After removing the cells by centrifugation at 8,000 rpm for 10 min at 4°C, the pH of cell-free supernatant was adjusted to 6.0 with 1M NaOH/1M HCl (Pringsulaka *et al*., 2012). The supernatant was heated to 80°C for 30 min for inactivation of extracellular proteases and hydrogen peroxide, and treated with proteinase K to confirm the production of bacteriocin like substance (Yi *et al*., 2010). The cell free supernatant was initially purified by 60% ammonium sulfate saturation at 4°C for 4 h (Ruiz-Martinez *et al*., 2013). After an additional centrifugation of 13,000 rpm for 30 min at 4°C, precipitates were re-suspended in 50 mM phosphate buffer (pH 7.0). The supernatant was filtered through 0.45 µm membrane filters (Pringsulaka *et al*., 2012). The filtrate was assayed to confirm antibacterial activity of active peptide against Gram positive and negative bacteria before performing chromatography techniques for further purification.

Antibacterial activity assay

The antibacterial activity of cell free supernatant was assayed through agar well diffusion method (Hata *et al*., 2010). To achieve this, nutrient agar plates were inoculated with approximately 10^5 CFU/mL of indicator strains (*Staphylococcus aureus* ATCC 29923, *Bacillus subtilis* JS-2004, *Bacillus cereus* ATCC 14579, *Streptococcus mutans* ATCC 25175, *Escherichia coli* ATCC 29922, *Pasteurella multocida* ATCC 51687). Later, small wells of 8 mm diameter were punched in hardened agar plates and filled with cell free supernatant and incubated overnight at 37°C. The diameter of inhibition zone (mm) around the wells was measured with the help of zone reader (Abbasi *et al*., 2013; Iram *et al*., 2013).

Bacteriocin purification

The homogenized purification of bacteriocin was performed by using cation exchange column of Carboxymethyl (CM) cellulose. The column was equilibrated with 50 mM sodium acetate buffer (pH 5.0) and washed subsequently with buffers A (50 mM sodium acetate buffer (pH 5.0), 200 mM NaCl), B (50 mM sodium acetate buffer (pH 5.0), 400 mM NaCl), C (50 mM sodium acetate buffer (pH 5.0), 600 mM NaCl, D (50 mM sodium acetate buffer (pH 5.0), 800 mM NaCl) and buffer E (50 mM sodium acetate buffer (pH 5.0), 1000 mM NaCl) respectively. Proteins were eluted at a flow rate of 1 mL/min in 1.5 mL fractions (Deraz *et al*., 2005). Antimicrobial activity of all fractions was measured by zone reading as described above. The

active fractions from ion exchange chromatography containing bacteriocin peptide were pooled together and loaded on Sephadex G-200 column, equilibrated with 30 mM phosphate buffer (pH 7.5; Aslam *et al*., 2012). The fractions were eluted at a flow rate of 0.5 mL/min. The collected elutions were passed again through antibacterial activity assay.

Effect of bacteriocin on biofilm formation

The effect of bacteriocin on biofilm formation was assayed according to the method of Srdjan et al. (Srdjan *et al*., 2000). The wells of a sterile 24 well flat bottomed microtiter plate were filled with crude and purified bacteriocin of *L. acidophilus* to observe biofilm inhibition. Twenty five and 50% percent (v/v) of bacteriocin suspension were added in total 1ml of nutrient broth and inoculated with 100 µL bacterial culture of *B. cereus*. Negative control well contained broth and bacterial culture only, and the positive control contained rifampicin instead of bacteriocin suspension. The plates were covered and incubated aerobically for 24 h at 37°C. After that, the content of each well were rinsed three times with 250 µL of sterile 0.9% NaCl and vigorously shaken in order to remove all non adherent bacteria. The remaining adherent bacteria were fixed with 200 µL of 99% methanol per well, and after 15 min the plates were emptied and left to dry. Then, the plate was stained for 5 min with 0.2 mL of 2% crystal violet per well. Excess stain was rinsed off by placing the plate under running tap water and air dried. The dye bound to the adherent cells in well were re-solublized with 160 µL of 33% (v/v) glacial acetic acid per well. The OD of each well was measured at 630 nm using ELISA reader (Srdjan *et al*., 2000). All the tests will be carried in triplicates, and the bacterial growth inhibition (INH%) was calculated by using the formula: INH% =100 – $(OD_{630\ sample} \times 100)/OD_{630\ control.}$

Microscopic evaluation

The inhibition activity of bacteriocin on biofilm formation was also observed under microscope. To obtain this, the overnight culture of *B. cereus* was added in few drops of nutrient broth on glass slide and incubated for 14 h at 37°C. The slides were washed with phosphate buffer saline and supplemented with crude and purified bacteriocin suspensions after incubation. After that, rinsing, staining and dissolving of biofilm were done according above mention procedure (Srdjan *et al*., 2000). For all assays, a negative control slide without bacteriocin and a positive control slide with rifampicin instead of bacteriocin suspension were prepared. The prepared slides were examined microscopically.

Statistical analysis

Statistical analysis was performed by taking mean and standard deviation of the three individual experiments and an analysis of variance (ANOVA) was applied and $p < 0.05$ was considered statistically significant.

RESULTS

Antibacterial activity assay of identified LAB specie

The identified LAB specie was confirmed as *Lactobacillus acidophilus* by using API 50 CHL system. The bacteriocin produced by

Table 1. Antibacterial activity against Gram positive and negative bacteria

Indicator strain	G+/G-	Diameter of Inhibition Zone (mm)	Bcateriocin activity (AU/mL)
Staphylococcus aureus ATCC 29923	G+	20 ± 0.2	2640.00
Bacillus subtilis JS-2004	G+	20.5 ± 0.1	2799.16
Bacillus cereus ATCC 14579	G+	20 ± 0.5	2640.00
Streptococcus mutans ATCC 25175	G+	18.5 ± 0.5	2186.31
Escherichia coli ATCC 29922	G-	14 ± 0.4	1037.20
Pasteurella multocida ATCC 51687	G-	15 ± 0.2	1265.06
Positive control (Rifampicin)		24 ± 0.2	4022.91

ATCC: American type culture collection, G+ = Gram positive bacteria, G- = Gram negative bacteria. Data are presented as means ± SD (n = 3); the mean difference is statistically significant at 95% confidence level (p = <0.001).

identified LAB exerted strong inhibition activity against Gram positive bacteria as compared to Gram negative bacteria in agar well diffusion assay, shown in Table 1.

Characterization of bacteriocin

First of all the activity titre of bacteriocin was concentrated by salt precipitation using ammonium sulfate to 4404.25 AU/mL. In the following purification step, the CM cellulose cation exchanger yielded maximum activity in NaCl concentration range of 0.4-0.5M. These fractions yielded 1.6% bacteriocin yield with 15.9 purification fold from culture supernatant and these fractions were considered for further purification. The highest loss of bacteriocin occurred during this step of purification. In the last step of purification, sephadex G-200 column purified bacteriocin up to 51 folds but resulted in 1.1% recoveries. The overall activities and yield are given in Table 2. The active fractions of ion exchange and gel filtration were run on SDS PAGE and the expected molecular mass of bacteriocin was found to be 6.5 kDa (Fig. 1).

Effect of inhibition activity of purified bacteriocin against biofilm formation

The inhibitory peptide exhibited strong antibacterial activity against Gram positive bacteria, therefore the purified fractions were further investigated only to observe the inhibition of biofilm by *B. cereus*, a food borne pathogen. The bacteriocin extract obtained after subsequent purification steps, namely, ammonium sulfate precipitation, ion exchange chromatography and gel filtration chromatography were examined against biofilm formation by *B. cereus*. The 50% (v/v) treatment of each purification fraction exhibited strong bacterial growth inhibition as compared to 25% (v/v) treatment. Meanwhile, each purification fraction of bacteriocin was used in

Table 2. Purification folds and recovery values of *L. acidophilus* bacteriocin in subsequent purification steps

Fractions	Vol.[a] (mL)	Protein conc.[b] (mg/mL)	Activity (AU/mL)	Total activity (AU)	Specific Activity (AU/mg)	Purif.[c] fold	Yield (%)
Culture supernatant	500	11.6	2799.06	1399530	214.3	1	100
i. Ammonium sulfate ppt.[d]	100	4.4	4404.25	440425	1000.9	4.7	31.5
ii. CM cellulose (cation exchanger)	6	1.1	3741.40	22448.5	3401.3	15.9	1.6
iii. Sephadex G-200 (Gel filtration)	3	0.44	4804.60	14413.8	10919.6	51	1.1

[a]Volume, [b]protein concentration, [c]purification fold, ammonium sulphate precipitation 60%. The protein concentration was calculated by measuring absorbance at 595 nm using Bradford reagent. Activity unit (AU) is the unit area of inhibition zone per unit volume (mm^2/mL); Specific activity (AU/mg), total activity of the subsequent purification step/total protein of the same step; Yield (%), total activity of subsequent step x 100/total activity of crude preparation; Purification fold, specific activity of subsequent step/specific activity of crude preparation.

Table 3. Biofilm inhibition by bacteriocin of *L. acidophilus*

Bacteriocin extracts	INH% of bacteriocin (25% v/v treatment)	INH% of bacteriocin (50% v/v treatment)
Culture supernatant	38.32	65.01
Ammonium sulfate ppt. extract	50.74	72.79
Ion exchange extract	58.29	87.12
Gel filtration extract	60.20	96.42
Positive control	89.0	89.0
Negative control	0	0

INH%: bacterial growth inhibition, positive control: rifampicin, negative control: bacterial growth inhibition in the absence of bacteriocin.

comparison with culture supernatant, negative control without bacteriocin extract and positive control with rifampicin. The bacterial growth inhibition (INH%) was calculated by measuring OD at 630 nm in flat bottomed microtiter plate, given in Table 3. A gradual increase in bacterial growth inhibition was observed with subsequent steps of purifications as compared to culture supernatant of crude extract. Whereas, the highly purified fraction of gel filtration exerted strongest inhibition activity against biofilm formation, which was further confirmed by microscopy

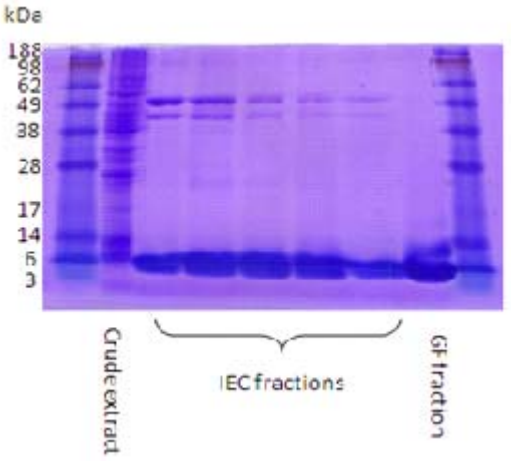

Fig. 1. Tris-glycine SDS-PAGE of crude supernatant and purified bacteriocin of *L. acidophilus*. Gel stained with Coomassie brilliant blue (R-250). Culture extract of *L. acidophilus* in lane 2 from left to right. Lane 3-7 have ion exchanged purified fraction and lane 8 has gel filtration purified fraction of *L. acidophilus* bacteriocin with estimated molecular mass of 6.5 kDa. Marker: see blue plus 2 protein standard.

Microscopic observations

The above mentioned results showed that the highly purified gel filtration extract of 50% (v/v) exhibited the highest antibiofilm activity. Thus, a microscopic analysis was carried out to further investigate the inhibition activity of only 50% concentration treatment of subsequent purification steps against biofilm formation of *B. cereus*.

DISCUSSION

The antibacterial peptide produced by *L. acidophilus* falls in the category of class II bacteriocins. Such bacteriocins exhibited barrelstave-like pore formation or a carpet mechanism of action against other bacteria. In this mechanism, the peptides of bacteriocin oriented parallel to the membrane surface and interfere with membrane structure (Moll *et al*., 1999). The cell free supernatant or crude extract of bacteriocin was ajdusted to pH 6 with 1M NaOH to eliminate the effect of lactic acid on inhibition of test strains. The sample was further heated to 80°C for 30 min to exclude the effect of extracellular proteases and hydrogen peroxide. The activity of bacteriocin like substance was confirmed by treating with

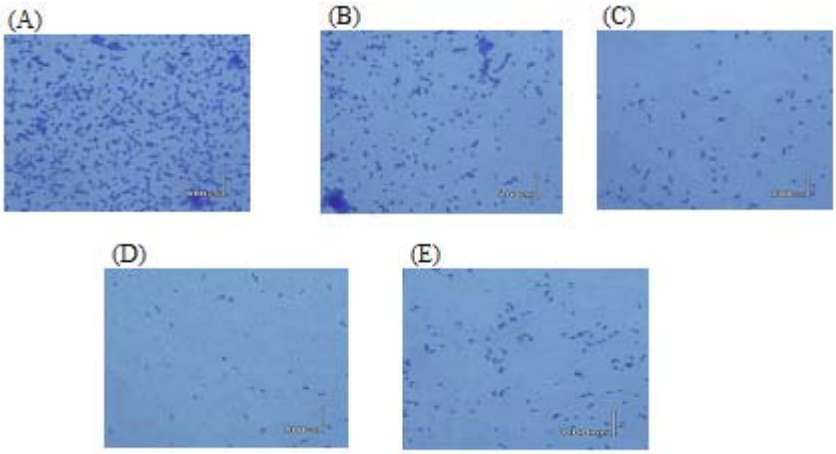

Fig. 2. Gradual inhibition of *B. cereus* biofilm formation in subsequent steps of bacteriocin purification, under 100 x magnifications. (A) Native growth slide of *B. cereus*. (B) Partially purified bacteriocin by ammonium sulfate precipitation. (C & D) The slides of biofilm treated with purified bacteriocin by ion exchnage and gel filtration chromatography respectively and depicted a successful antibiofilm activity by inhibition. (E) Positive control (rifampicin).

proteinase K which totally eliminated its activity. So, it was confirmed that the antibacterial activity of supernatant was due to the presence of bacteriocin (Yi *et al*., 2010).

The resulted 6.5 kDa peptide in the present study has strong antagonistic activity against Gram positive food borne pathogens as compared to Gram negative bacteria in antibacterial activity assay. Moreover, the measure of zone of inhibition around a well or spot was considered as a most simple or direct method of bacteriocin detection (Chen and Hoover, 2003). Meanwhile, the activity against many Gram-negative bacteria was not frequently seen by bacteriocins of LAB. The bacteriocin isolated from *L. acidophilus* AA11has wide inhibitory activity against Gram positive bacteria (*B. subtilis, B. cereus and S. aureus*) and Gram-negative pathogens (e.g., *E. coli*, *Salmonella* sp. and *Shigella* sp.; Abo-Amer, 2011). Whereas, a 6.6 kDa acidocin D20079 isolated from *L. acidophilus* DSM 20079 is quite sensitive and its antibacterial spectrum is restricted to some *Lactobacillus* sp. including *L. delbrueckii* ssp. *lactis* DSM 20076, *L. bulgaricus* DSM 20080 and *L. sakei* NCDO 2714 (Deraz *et al*., 2005).

The biofilm formation depends on an interaction between bacterial cells, the attachment surface and the surrounding medium (Chang *et al*., 2013; Lou *et al*., 2013). The biofilm formation by *L. acidophilus* in present study successfully inhibited the growth of Gram positive test strains by exerting bactericidal effect, and considered as a good antibiofilm agent to be used in food sectors.

The resulted bacteriocin should also be used against the formation of biofilm by *S. mutans*, which is considered as a major problem in dentistry. Similarly, different species of bacteriocins producing LAB, *E. faecium* and *V. carniphilus* exhibited high antagonistic activity against biofilm formation of *H. alvei*, *S. aureus* and *L. innocua* whereas, *L. sakei* did not showed any positive influence on biofilm inhibition (Ammor *et al*., 2006). In another reported study, the antagonism of *L. lactis* producer of bacteriocin nisin was investigated against biofilm formation of *L. monocytogenes*. The results of this investigation predicted that the *L. lactis* exhibited antimicrobial effect against biofilm of *L. monocytogenes* but hydrolysis/inhibition of biofilm depends on inoculum size of indicator strain (Leriche *et al*., 1999). Another strain of *L. lactis* UQ2 isolated from Mexican-style cheese was investigated against *L. monocytogenes*. The experiment was conducted on direct incorporation of *L. lactis* UQ2 inoculum and the spray-dried crude bacteriocin fermentate of this strain. They suggested that the combined effect of nisin and lactic acid produced by *L. lactis* UQ2 were found to be more effective against *L. monocytogenes* growth than each one on its own (Garc1a-Almendarez *et al*., 2008).

Similarly, the bacteriocin produced by *L. acidophilus* showed good examination of biofilm formation inhibition of food borne pathogen *B. cereus* at 50% concentration treatment (Figure 1). Moreover increasing concentration of bacteriocin displayed increased antibiofilm activity even against other Gram positive pathogenic bacteria like *Staphylococcus aureus* and *Listeria monocytogenes*, which are also the predominant food borne pathogens and causative biofilm agents.

CONCLUSION

Majority of lab work have been conducted on the conclusive effect of lactic acid producing bacteria (LAB) on growth inhibition of pathogenic bacteria or the effect of natural antimicrobial peptide on spoiling or disease causing microbes. In present study, a low molecular mass bacteriocin produced by *L. acidophilus* has strong antibacterial activity not only against Gram positive but also against Gram negative bacteria. Indeed, these antibacterial peptide displayed strong antagonism effect against the growth of indicator strain, *B. cereus* and should also be used as antibiofilm agent against other food spoiling bacteria.

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