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EVALUATION OF CYTOTOXICITY AND CHARACTERIZATION OF BACTERIOCINS OF POTENTIAL PROBIOTIC ISOLATES FROM CATTLE FARM SOIL OF DIBRUGARH DISTRICT, ASSAM, INDIA

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ABSTRACT

Antimicrobial property and safety are important features in the screening of probiotic isolates. The study aims to evaluate the safety and characterize the antimicrobial activity of crude bacteriocins of newly isolated potential probiotic bacterial strains namely *E. faecium* DUD3, *Bacillus sp* DUA4 and *Enterobacter sp* DUE2 from cattle farm soil in Dibrugarh District, Assam, India. Cytotoxicity test of the crude bacteriocin of the isolates against HEK293 and HT29 cell lines was evaluated using MTT assay and the result showed survival rate of more than 50% for both the cell types following treatment with bacteriocin of *E. faecium* DUD3. However, the survival rate of these cell types was found be less than 50% following treatment with *Bacillus sp* DUA4 and *Enterobacter sp* DUE2. Characterization of the crude bacteriocin of the isolates was studied using assay of the residual antimicrobial activity following treatment with proteolytic enzymes and at different temperature and pH. The crude bacteriocin of *E. faecium* DUD3 was found to be sensitive to all proteolytic enzymes tested and showed significant resistance to a wide range of pH and temperature. In the present study, out of the three isolates, *E. faecium* DUD3 was found to be most potential probiotic candidate followed by *Bacillus sp* DUA4.

KEYWORDS: Cytotoxicity, MTT, Bacteriocin, *E. faecium*, *Bacillus spp.*

INTRODUCTION

The antagonistic effect of probiotics may be either as a result of direct effect of the probiotic organisms or by the production and release of metabolites mainly bacteriocins directed

against target organisms. Stimulation of the host immunity by probiotic and normal flora is also indirectly involved in the protection against harmful organisms.[1] In the last few decades, interest in bacteriocin of probiotics has grown steadily as they can be potentially applied as biopreservatives, in food and food products.[2] Bacteriocin can be produced by bacteriocinogenic strains of both gram positive and gram negative bacteria. Considering their long traditional use in food, bacteriocins produced by Lactic Acid Bacteria (LAB) are increasingly becoming important potential "biopreservative". Bacteriocinogenic enterococci are increasingly becoming important as they are frequently isolated from food materials or silage with significant activity against pathogenic and food spoilage organisms and specially *Listeria* and *Clostridium* species.[3] This has led to the practical applications of enterocins or the enterocin-producing strains as biopreservatives and are applied in the form of a starter culture or coculture.[3,4]

Enterococci and bacillus species are frequently isolated from the environment, food and clinical specimens with *Enterococcus faecium, Enterococcus faecalis* and *B subtilis* being predominant and are being used as probiotics and are also used in the processes of food fermentation giving essential benefit to human being and animals.[3] However, some strains of enterococcus by virtue of possessing virulence determinants and transferrable antibiotic resistance may result in nosocomial infections especially among immunocompromised individuals.[5,6] Bacillus species are industrially important and have been applied in food industries. *B subtilis* which is used in the preparation of Natto, fermented food in East Asia is a common example.[8,7] Though most of the probiotic bacteria are historically known to be associated with food and are safe for consumption, it is mandatory to conduct standard safety evaluation of any potential probiotic candidate as part of screening process. The objective of this study was to evaluate the safety and study the characteristics of the crude bacteriocin extract of three newly isolated potential probiotic bacteria namely *Enterococcus faecium* DUD3 (Accession number KT340075), *Bacillus sp* DUA4 (Accession number KT340076) and *Enterobacter sp* DUE2 (Accession number KX097968) from cattle farm soil of Dibrugarh District, Assam, India.

MATERIALS AND METHODS

Production of bacteriocin

Production of bacteriocin was done using de Man, Rogosa and Sharpe (MRS) broth by inoculating freshly grown colonies of the selected isolates followed by overnight incubation

at 36°C and shaking condition with the speed of 136 rpm. Harvesting of Cell Free Culture Supernatant (CFCS) was done by centrifugation of the culture broth at 15,000g at 4°C for 10 minutes. Isolation of crude bacteriocin from CFCS was done by cold acetone precipitation at a ratio of 1:1.[9] Harvest of crude bacteriocin precipitate was done by centrifugation at 25,000g at 4°C for 10 minutes. Solution of the bacteriocin precipitate at the desired concentrations was prepared for the assays conducted.

Safety evaluation

Safety evaluation of the partially purified bacteriocin of the isolates was done using *in vitro* viability assay. For this purpose MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay was conducted as described by Mossman using cell lines namely Human Caucasian colon adenocarcinoma (HT29) and Human Embryonic Kidney cells 293 (HEK293) as indicators of cytotoxicity.[10] The HEK293 and HT29 cells grown to 80% confluence was treated with different concentrations of the crude bacteriocin extract of *E. faecium* DUD3, *Bacillus sp* DUA4 and *Enterobacter sp* DUE2 for 24 hours under optimal growth condition. Detection of the viability of the cells was done by addition of MTT and taking Optical Reading (OD) at 570 nm following incubation. The OD reading of the control was used as a reference to calculate the percent cell viability using the following formula.[11]

(Optical density of the test sample/optical density of the negative control) × 100

Characterization of bacteriocin

Detection of antimicrobial activity

Animicrobial activity assay for the isolates that showed better safety level namely *Enterococcus faecium* DUD3 and *Bacillus sp* DUA4 was conducted using crude bacteriocin extract dissolved in neutralized and filter sterilized CFCS of the respective isolates at a concentration of 50mg/ml using well diffusion method.

Resistance to high temperature

The assay for the heat resistance of the partially purified bacteriocin obtained from the isolates *Enterococcus faecium* DUD3 and *Bacillus sp* DUA4 was done using a previously described method with little modification.[12] One ml of the bacteriocin suspension of the isolates was heated at 60, 80, 90, 100, 121°C for 15 and 30 minutes. Heat resistance of the bacteriocin was verified by checking for the residual activity against *L. monocytogenes* using well diffusion assay and activity was compared with that of untreated crude bacteriocin.

Stability at different pH

Stability of the crude bacteriocion of the isolates *Enterococcus faecium* DUD3 and *Bacillus sp* DUA4 was done using a method previously described.[13] Crude bacteriocin of the isolates was exposed to different pH conditions in the range of 3 to 10 adjusted using 1N HCl and 1N NaOH for 2 hours. Residual activity of the bacteriocin was assayed using well diffusion method following pH adjustment to 6.5.

Resistance to enzymes

The crude bacteriocin of the isolates was tested for susceptibility to pepsin (LOBA CHEMIE, 1:3000), proteinase K (HiMedia, >= 30 U/mg protein) and papain (HIMEDIA, 30000 USP Units/mg) using previously described method.[14] Each of the enzymes was dissolved in PBS adjusted to pH 7.0 except in the case of pepsin where it was adjusted to pH 4.0. and desired amount of the solution was mixed with the crude bacteriocin solution to give a final concentration of 1 mg/ml. The enzymatic treatment was done by incubation of the mixture at 36°C for two and half hours. At the end of incubation time the solution was heated at 80°C for 10 minutes to stop further enzymatic action. Moreover the solution for pepsin treatment was neutralized using NaOH. Detection of residual antimicrobial activity following treatment was done using well diffusion technique. Untreated crude bacteriocin was used as a positive control.

RESULT AND DISCUSSION

Safety evaluation

The result for cytotoxicity assay as it is given in Table 1 shows that more than 50% survival value of HT29 cells was obtained following treatment with *E. faecium* DUD3 at all concentrations tested. However, for the same cell line treated with crude bactriocin of *Bacillus sp* DUA4 and *Enterobacter sp* DUE2, survival value of more than 50% of the cells was only observed at a concentration of 25 mg/ml or less of crude bacteriocin. On the other side the survival rate of HEK293 cells following treatment with different concentrations of the crude bacteriocin of the isolates *E. faecium* DUD3, *Bacillus sp* DUA4 and *Enterobacter sp* DUE2 showed low survival rate. Survival rate of more than 50% was only observed with the isolate *E. faecium* DUD3 at all the concentrations tested except at the concentration of 100 mg/ml.

Table 1. Cell viability of HEK293 and HT29 cell lines following treatment with crude bacteriocin of potential probiotic isolates.

Isolates	Concentration (mg/ml)	Viability (%)					
		Cell lines					
		HEK293			HT29		
Control	-	100.000	±	0.068	100.000	±	0.009
E. faecium DUD3	100	30.690	±	0.014	57.610	±	0.004
	50	54.630	±	0.009	73.650	±	0.001
	25	70.490	±	0.072	97.230	±	0.003
	12.5	70.700	±	0.036	98.350	±	0.004
Bacillus sp DUA4	100	9.540	±	0.028	48.620	±	0.006
	50	11.480	±	0.018	48.750	±	0.003
	25	27.850	±	0.029	70.390	±	0.005
	12.5	48.300	±	0.049	91.930	±	0.006
Enterobacter sp DUE2	100	9.940	±	0.016	45.650	±	0.001
	50	10.120	±	0.015	47.400	±	0.002
	25	13.720	±	0.007	57.150	±	0.002
	12.5	31.860	±	0.054	84.190	±	0.004

In general the survival rate of HT29 cells following treatment with crude bacteriocin of the isolates *E. faecium* DUD3, *Bacillus sp* DUA4 and *Enterobacter sp* DUE2 was higher than that of HEK293 treated under the same conditions. Moreover, the overall comparison of the survival rate of the cells following treatment with crude bacteriocin shows the relative toxicity of the isolates and such a comparison as it is given in Figure 1 shows that survival rate of more than 50% for both of the cell lines applied at the concentration of 50 mg/ml was only observed with *E. faecium* DUD3. The result suggested that *E. faecium* DUD3 followed by *Bacillus sp* DUA4 to be potentially safe isolates.

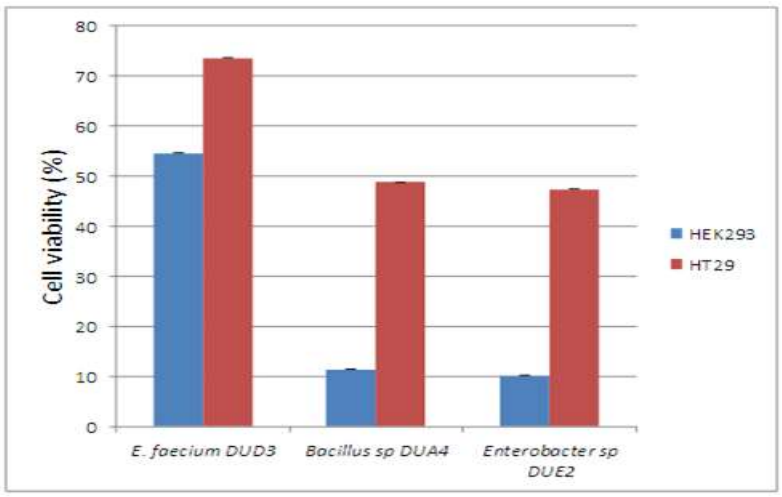

Figure 1. Comparison of cell viability of the cell lines HEK293 and HT29 treated with crude bacteriocin of potential probiotic isolates at a concentration of 50 mg/ml.

On the other hand, the isolate *Bacillus sp* DUA4 has shown considerable level of safety specially as applied against HT29 cell line. In another similar study conducted using MTT assay on bacillus species, a contrasting result was obtained for the different strains tested. Low level cytotoxicity was found with *B. subtilis* while *B. cereus* strain was found to be highly toxic to the cell lines tested.[15] The survival rate of the cells treated with *Enterobacter sp* DUE2 for both the cell lines was found to be the lowest and as a result the isolate was not considered for further characterization.

Antimicrobial activity

The antimicrobial activity of the crude bacteriocin of the potential isolates against the tested organisms (Table 2) indicated that both *Enterococcus faecium* DUD3 and *Bacillus sp* DUA4 showed activity against *B. subtilis* (ATCC 6051), *S. epidermidis* (MTCC 6810), *L. monocytogenes* (ATCC BAA-751) and *B. cereus* (ATCC 11778). However, activity against *S. aureus* (MTCC 9542) was only observed with *Enterococcus faecium* DUD3 but not with *Bacillus sp* DUA4. The result in this study was found to be in partial agreement with the broad spectrum activity of E. *faecium* 130 which inhibited *L. monocytogenes* and some other gram positive bacteria, but not against the *Staphylococcus aureus* strain tested.[16] In another study, the bacteriocin produced by *E. faecium* MMT21 inhibited not only closely related LAB, but also *L. monocytogenes* and *S. aureus*.[17]

Table 2. Antimicrobial activity of crude bacteriocin extract of potential probiotic isolates obtained from cattle farm soil against indicator organisms.

Indicator organisms	Diameter zone of inhibition	
	***Bacillus sp* DUA4**	***E. faecium* DUD3**
B. subtilis (ATCC 6051)	+	+
S. epidermidis (MTCC 6810)	+	+
L. monocytogenes (ATCC BAA-751)	+	++
B. cereus (ATCC 11778)	++	+
S. aureus (MTCC 9542)	-	++

Key: ++ =14 to 19 mm, + = 11 to 14 mm, - =11mm.

3.3 Characterization of bacteriocin

Temperature and pH stability and resistance to enzymatic treatment is essential characteristics of probiotics both in the processing and application of probiotics.[18] The result for the assays of stability of antimicrobial activity of the crude bacteriocin of the isolates following treatment with proteolytic enzymes and at different temperature and pH is given in

Table 3. With regard to the stability of crude bacteriocin towards enzymatic treatement, extract of both *Enterococcus faecium* DUD3 and *Bacillus sp* DUA4 showed sensitivity to proteolytic enzymes as indicated by the loss of activity after treatment with the respective enzymes. Bacteriocin sensitive to proteolytic enzymes are broken down by gastric juices and this contributes towards its safe nature for human consumption. Enzyme treatment of crude bacteriocin obtained from *Enterococcus faecium* DUD3 showed complete absence of antimicrobial activities after treatment with the enzymes pepsin, proteinase K and papain.

Table 3. Effect of proteolytic enzymes, pH and temperature on the antimicrobial activity of crude bacteriocin of potential probiotic isolates obtained from cattle farm soil.

Isolates	Antimicrobial activity after treatment	
	***Bacillus sp* DUA4**	***Enterococcus faecium* DUD3**
Resistance to enzymes		
Papain	+	-
Pepsin	-	-
Proteinase K	-	-
Control	+	+
Resistance to different pH		
pH 3	-	-
pH 4	-	+
pH 5	-	+
pH 6	+	+
pH 7	+	+
pH 8	+	+
pH 10	-	-
Control	+	+
Temperature (Time in minutes)		
60^{o}C (15)	+	+
(30)	+	+
80^{o}C (15)	+	+
(30)	+	+
90^{o}C (15)	+	+
(30)	+	+
100^{o}C (15)	+	+
(30)	-	+
121^{o}C (15)	-	+
Control	+	+

Similar finding was also reported for the inhibitory activity of the bacteriocin produced by *E. faecium* JCM 5804T where the antimicrobial activity was lost following treatment with proteolytic enzymes such as proteinase K, trypsin and papain.[19]

In another similar study, loss of antimicrobial activity of culture supernatant of *E. faecium* strains namely MMT21 and strains of *E. faecalis* was observed following treatment with proteolytic enzymes.[17,20,21,22] Crude bacteriocin obtained from *Bacillus sp* DUA4 was found to be sensitive to proteinase K and pepsin but was resistant to papain. Bacteriocin obtained from potential probiotic isolates *Bacillus licheniformis* ZJU12 and *Bacillus amyloliquefaciens* showed sensitivity to the treatment with proteolytic enzymes as indicated by the loss of antimicrobial activity against the indicator organism used.[23,24] The same bacteriocin obtained from *Bacillus sp* DUA4 in this study was resistant to papain and this was in agreement with the finding in other study.[25]

With regard to the stability of the crude bacteriocin of the isolates in relation to the change in pH, both *E. faecium* DUD3 and *Bacillus* sp DUA4 were not able to tolerate a wide range of pH and antimicrobial activity was only observed in the pH range of 4-8 and 6-8 for the isolates *Enterococcus faecium* DUD3 and *Bacillus sp* DUA4 respectively. In another study, the antilisterial activity of the culture supernatant of three E *faecium* strains was found to be stable in a pH ranging from 3.0-10 showing more stable nature than that of the activity of the isolates in the current study.[21] Moreover, contrary to the finding in this study, CAMT2 bacteriocin obtained from *Bacillus amyloliquefaciens* showed stability under wide range of pH ranging from 2-10.[24]

Crude bactriocin extract of *Enterococcus faecium* DUD3 was found to be stable at high temperature even following incubation at 121^{o}C for 15 minutes. Different bactriocins such as enterocin BFE 900 and enterocin MR99 produced by other strains of *E. faecium* has also been reported to show similar level of heat resistance.[22,26] The stability of the crude bacteriocin of Bacillus *sp* DUA4 to high temperature was found to be such that the crude bacteriocin lost activity following exposure to 100^{o}C for 15 minutes. Culture supernatant obtained from *Bacillus subtilis* R75 however, was observed to be more heat stable and was found to maintain its antimicrobial activity even after exposure to 121°C for 15 minutes.[27]

CONCLUSION

In this study out of the three potential probiotic isolates obtained from cattle farm soil, *E. faecium* DUD3 was found to be the most potential probiotic candidate followed by *Bacillus sp* DUA4 based on the evaluation of cytotoxicity and characterization of bacteriocin.

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