IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB)
ISSN: 2455-264X, Volume 3, Issue 2 (Mar. – Apr. 2017), PP 28-33
www.iosrjournals.org

Bacteriocin: Promising Natural Antimicrobials from Probiotic Bacillus subtilis

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Abstract: *The beneficial effects of probiotics on host have been studied and explored for over a century. The aim of this research work was to investigate the maximum bacteriocin production from probiotic bacteria Bacillus subtilis resistance to low pH and bile salt and extraction of bacteriocin by using Chloroform extraction method.*

The antimicrobial activity was carried out using agar well diffusion method against pathogens such as E.coli, S.aureus, Aspergillus niger, Candida albicans. Maximum inhibition zone was shown by S.aureus about 21±0.85.Bacteriocin susceptibility testing been carried out based on broth dilution method described by CLSI guidelines. The MIC of bacteriocin against S.aureus range was in between 128 to 64mg/l and MBC value was 96 mg/l. The antioxidant potencial of the cell free extract (crude) and partially purified bacteriocin of the probiotic isolate were investigated using DPPH scavenging activity assay.It showed moderate antioxidant activity by inhibiting 53% and 62% inhibition of free radical respectively. Using ascorbic acid as positive control. By using FTIR spectroscopy characterization of Bacteriocin was done. FTIR results showed that the bacteriocin was peptide based compound.

Key words: *Probiotics, Bacteriocin, MIC, MBC, FTIR.*

I Introduction

The term probiotics was derived from the Latin word "pro" which means "for" and the Greek word "bios" which means "life".At the beginning of 20th century, Elie Metchnikoff a scientist at the Pasteur institute was the first who described the concept of probiotics. He proposed that the acid producing bacteria in fermented milk products could prevent fouling in the large intestine and if consumed regularly, lead to a longer, healthier life[1,2] . Henry Tisser a French paediatrician, during his working he isolated 'Y' shaped bacteria- *Bifidobacteria* from the stool samples of children who were suffering from the diarrhea. He suggested that these bacteria could be administrated to patients with diarrhea to help restore a healthy gut flora [3,8]. In 1965, the Lilly and Stillwell was the first who coined up term probiotic. They described probiotic as a "substances secreted by one organism which stimulate the growth of another" [4]. Parker defined probiotic as organisms and substances, which contribute to intestinal microbial balance" [5]. Salminen et al in 1999 defined probiotic as "food containing live bacteria which are beneficial to health[6].There are numbers of definitions have been proposed and used by researchers over a century but as per FAO/WHO (2002) Probiotics defined as "live microorganisms which when administrated in adequate amounts confers health benefits on host" which have been used widely[7,10].

Probiotics are viable cells which has beneficial effects on host by altering or improving its intestinal flora[9]. There are several microorganisms which are claimed as probiotics, its includes *Lactobacillus, Lactococci, Bifidobacterium and Saccharomyces.*Other probiotic bacteria received less attention of researches which involves bacteria of *Bacillus* genus[12]. *Bacillus* genus is a heterogenous group of Gram positive, facultative anaerobic,endospore forming rod shaped bacteria, which includes extremophiles and mesophiles. They are widespread microorganism in nature, they can be found in soil, water and air.*Bacillus* bacteria produces large amount of antimicrobial compound and enzymes which are having broad inhibitory spectrum against pathogenic bacteria and fungi [11,13].

Bacillus subtilis the member of bacillus is one of the most important commercially used probiotics. Bacteriocins are one of the antimicrobial compound synthesized by probiotic bacteria *Bacillus subtilis* as a secondary metabolite. Bacteriocins are defined as ribsomally synthesized small polypetides that exerts antimicrobial effect against closely or non related bacteria[15,16]. According to FDA (Food and Drug administrated *B.subtilis* is Generally Recognized as a Safe(GRAS). In this sense, *B.subtilis* may be considered as a probiotic bacterium for humans and animals[14].

The probiotic bacterium should possess essential characteristics to be an ideal probiotic. There are some criteria which have been used to check whether the microorganism is suitable as a probiotic or not. They have to pass through from the stressful conditions of stomach to reach small intestine. However, the pH of stomach can be as low as 2. Not only pH but high temperature of stomach also decreases the viability of probiotic bacterium. Hence before the microorganisms used as a probiotic, it must fulfill some criteria related to

stability in adverse conditions and safe to host. Bacteriocin production is also considered as one of the important criteria in the selection of probiotic bacterium.
They should able to survive in wide range of pH and temperature, tolerance to gastric acid and bile juice.

Materials

Bacillus subtilis NCIM 2548 culture was procured from NCL, Pune and subcultured on nutrient agar slant. Inoculated slants were incubated at 37^0C for 24 hours. Indicator strains *E.coli* ATCC 25922 and *S.aureus* ATCC 25923 were subcultured on nutrient agar slant while *Aspergillus niger* NCIM 545 and *Candida albicans* MTCC 277 were subcultured on Sabourd agar slants. All the slants were stored at 4^0C till use.

II Methods

Essential probiotic properties of *Bacillus subtilis*

Resistant to low pH

To determine the effect of pH on bacterial growth, the 24 hours old culture of *Bacillus subtilis* NCIM 2548 were inoculated into the Modified TY medium (Tryptone - 10.0, Yeast extract 5.0, NaCl 5.0g/L) and adjusted to the different pH 3, 5, 7 and 9 adjusted with 1N HCl or 1N NaOH. Bacterial growth was monitored by determination of optical density at 600nm after every 6 hours.

Tolerance against Bile

Because the mean intestinal bile concentration is believed to be 0.3% (w/v) and the staying time of food in small intestine is suggested to be 4 hrs.The experiment was performed at this concentration of bile for 4 hrs. TY broth containing 0.3% bile (Oxoid) was inoculated with active culture (incubated for 24 hours). During the incubation for 4 hrs, growth was monitored as OD at 620nm.

Growth at Different Temperatures

Effect of temperature on bacterial growth was determined by using modified TY agar slants containing bromocresol purple indicator. Slants were streaked with loopful *Bacillus subtilis* NCIM 2548 suspension and incubated at 15,25, 35 and 45^0C. During incubation time bacterial growth at any temperatures was observed by the change of colour from purple to yellow.

Production and extraction of bacteriocin

Bacteriocin production by *Bacillus subtilis* NCIM 2548 was carried out in modified TY medium (Tryptone - 10.0, Yeast extract 5.0, NaCl 5.0g/L) having initial pH 7.0 and sterilized at 121^0C for 15 minutes. Inoculum (100ml) was grown in the medium at 37 ^{0}C for 48 hours.

Burianek L.L. and A.E Yousef used choloroform for the extraction of bacteriocin and suggested that it is one of the less time consuming and effective method which yields maximum amount of bacteriocin[16]. Following are the procedure have been used for extraction of bacteriocin.100ml of a modified T.Y broth was inoculated with 0.1% of an overnight culture of the *Bacillus subtilis* and incubated for 48 hrs at 37^0C.After 48 incubation the cells were pelleted at 8000 rpm for 15 min in a refrigerated (10^0C) centrifuge and the bacteriocin containing supernatant fluid (crude) was collected. Chloroform (50ml) was added to the supernatant fluid, stirred vigorously using a magnetic stirrer for 20 min, distributed into polypropylene bottles and centrifuged at 1000 rpm for 20 min. The sediments on the side and/or bottom and the solids at the interface, were recovered by carefull pouring off the top aqueous layer and holding back the floating interfacial precipitate at the interface with a pipette, then pushing back the interfacial precipitation while pouring off the solvent, which resulted in the interfacial precipitate remaining in the bottle. Tris buffer (0.1 mol l-1,5-10 ml, Tris,pH 7.0) was used to resuspend the contents of the bottles (sediments, interfacial precipitate and remainders of chloroform and culture medium) and the mixtures were combined in a 50ml Teflon tube. The combined mixture was centrifuged again at 12000 rpm for 15 min and sediments were separated from the remaining chloroform and medium. The pellet was transferred onto a small aluminium pan for drying in a chemical hood overnight[17,18].

Antimicrobial activity of Bacteriocin

The antimicrobial activity of crude and purified bacteriocin was determined by agar well diffusion method.The indicator bacteria were cultured on nutrient agar for 24 h at 37°C and used to prepare cell suspensions in normal saline. A lawn of the indicator strain was made by spreading the cell suspension over the surface of nutrient agar plate with a sterile cotton swab. The plates were allowed to dry and a sterile cork borer of diameter (6mm) was used to cut uniform wells in the agar. Each well was filled with 50µl of crude and purified bacteriocin separately. After incubation at 37^0C for 24 hrs, the plates were observed for a zone of inhibition (ZOI) around the well. The diameters of the inhibition zones around the wells were recorded in millimeters. Further purification of the crude bacteriocin was achieved through overnight dialysis in 5000 Dalton cutoff bags.

Determination of MIC and MBC

Antimicrobial compound susceptibility testing has been carried out based on broth dilution method described by CLSI guidelines. MIC of Partially purified bacteriocin against indicator organisms was determined by broth dilution method[19]. The MIC of indictor strain staphylococcus was determined in sterile Tryptic Soy broth. The colonies were suspended in 1 ml of sterile Tryptic soy broth and turbidity of suspension was set at 0.5 McFarland standard. The above inoculum was serially diluted by using sterile Mueller Hinton broth to get the final organism density 5 x 10^5cfu/ml (range 3-7 x 10^5cfu/ml). The partially purified bacteriocin has been diluted to concentration of 256.0,128.0 , 64.0, 32.0, 16.0 , 8.0, 4.0, 2.0, 1.0, 0.5, 0.25, and 0.125 mg/L respectively by using diluent and inoculated with indicator strain, inoculum prepared by using 0.5 McFarland standard. All tubes have been incubated at 37 for 24 hours. The visible growth of organism has been recorded. Results of MIC has been recorded after incubation in mg/l. The MBC was determined by diluting the partially purified bacteriocin between the MIC range and observe the minimum concentration at which the indicator organism growth was not observed were recorded[20].

Antioxidant activity

Antioxidant activity of the cell-free culture supernatant of *Bacillus subtilis* NCIM 2548 was determined by using the DPPH assay method [21,22] . 1, 1-diphenyl-2-picrylhydrazyl (DPPH) was prepared in ethanol (300 uM). 1ml of1,1-diphenyl-2-picrylhydrazyl (DPPH) was added 0.8 sample tubes and incubated at 37º C for 30 minutes in the dark. Absorbance was measured at 515 nm. Ascorbic acid was used as standard control[23]. The free radical scavenging activity was measured as percentage inhibition using following formula.

$$\% \text{ Inhibition} = \frac{(\text{Absorbance of the control} - \text{Absorbance of the test sample}) \times 100}{\text{Absorbance of the control}}$$

FTIR analysis

Characterization of partially purified powder was carried out by using SHIMADZU FTIR instrument. Analysis was done on an FTIR spectrometer.

III Results And Discussion

Resistance to pH

Being resistant to low pH is one of the major selection criteria for probiotic strains. Every 6 hours the experimental result was recorded which showed that at low pH there was maximum bacterial growth as compare to high pH. At pH 3,5 and 7 the bacterial growth was sufficient but decreased at pH 9.According to the observation the organism showed resistance to low pH while sensitive to high pH.

pH	**3**	**5**	**7**	**9**
OD at 600 nm	0.79±0.04	0.85±0.06	0.71±0.05	0.39±0.08

Tolerance against Bile salt

Another essential character of the probiotic organism is the tolerance to bile salt. The organism was also screened for their ability to tolerate the bile salt. The initial and final OD at 600 nm was 0.38 and 0.59 respectively. Thus it may be concluded that the organism tolerate and able to grow in 0.3% bile salt.

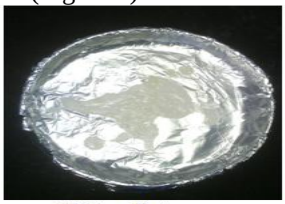
Growth at Different Temperature

It was found that the *B.subtilis* inoculated into modified TY agar slant and subjected to different incubation temperatures expressed the good activity at high temperature 35 and 45^{0}C, while showed less activity at low temperature 15 and 25^{0}C being optimum at 35^{0}C.

Production and Extraction of Bacteriocin

The culture of *Bacillus subtilis* was inoculated into the modified TY broth and incubated at 35^{0}C for 48 hours. After 48 hours it shows optimum production of bacteriocin. Maximum yield of bacteriocin was extracted by chloroform extraction method. After centrifugation the interfacial layer (pellet) were collected and was transferred into a small aluminium pan for drying in a chemical hood overnight. Partially purified powder of bacteriocin was preserved for further uses (Figure.1).

Figure.1 Bacterial growth



Wet pellet

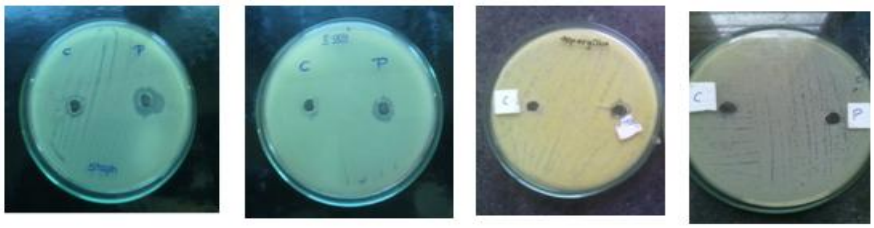
Partially purified bacteriocin powder

Antimicrobial activity of Bacteriocin

The antimicrobial activity of partially purified bacteriocin was carried out using agar well diffusion method. The inhibitory activity of cells free broth (crude) and Partially purified bacteriocin of *B.subtilis* was screened against indicator microorganisms such as *E.coli, S. aureus, A. niger and C.albicans*. Maximum inhibition zone was shown by *S.aureus*. Significant inhibition zones were shown by *E.coli,* and *A.niger*. (Table no.1 and Figure no.2)

Table no .1 Antimicrobial activity of bacteriocin against pathogens

	Pathogens Diameter of Inhibition Zones (mm)			
	E.coli	***S.aureus***	***Aspergillus***	***Candida***
Crude(culture free broth)	14±0.75	18±0.63	13±1.03	15±0.48
Partially purified bacteriocin	18±0.82	21±0.85	16±0.65	19±0.42



S.aureus ***E.coli*** ***A.niger*** ***C.albicans***

Figure no.2 Antimicrobial Activity of Bacteriocin against Pathogenic Organism

MIC and MBC

MIC was carried out of partially purified bacteriocin against indicator organisms. It showed that MIC value of partially purified organism against *E.coli* and *S.aureus* was in between same range 64mg/l and for *A.niger* and *C.albicans* the MIC value was in between 128mg/l (Table.2). The MBC values of partially purified bacteriocin for *E.coli* and *S. aureus* was found to be 80 mg/l whereas for *A.niger* and *C.albicans* it was 96 mg/l (Table.3).

Table 2. MIC value determination of bateriocin.

Concentration Mg/L	512	128	64	32	16	8	4	2	1
E.coli	- -	- -	- -	+	+ +	+ +	+ +	+ +	+ +
S. aureus	- -	- -	- -	+	+ +	+ +	+ +	++	+ +
A.niger	- -	- -	+	+ +	+ +	+ +	+ +	+ +	+ +
C.albicanes	- -	- -	+	+ +	+ +	+ +	+ +	+ +	+ +

(Growth= +, No growth= - -)

Table 3.MBC value determination of bacteriocin.

Concentration mg/L	128	112	96	80	64
E.coli	-	-	-	-	+
S.aureus	-	-	-	-	+
A.niger	-	-	-	+	+
C.albicanes	-	-	-	+	+

(Growth= + , No growth= -)

Antioxidant activity of Bacteriocin

Antioxidant activity was performed by using DPPH method. The antioxidant activity of cell free extract (crude) and Partially purified bacteriocin from *Bacillus subtilis* NCIM 2548 are carried out. It showed moderate antioxidant activity by exhibiting 53% and 62% inhibition of free radical. Ascorbic acid was used as standard control and it showed 87% inhibition of free radical.(Figure.3).

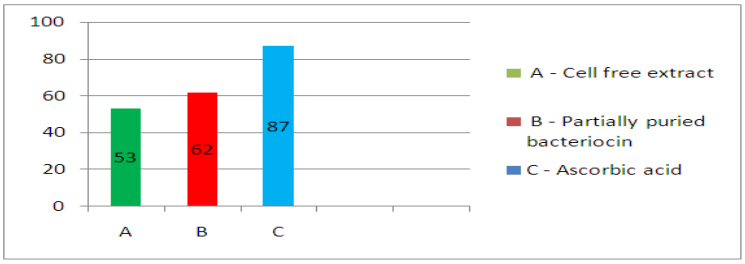
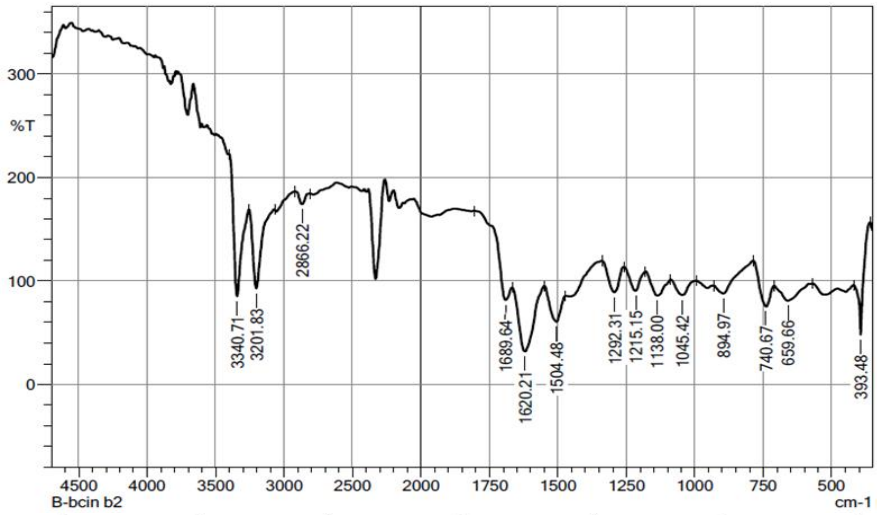


Figure.3 Antioxident activity of Bacteriocin.

FTIR analysis

FTIR analysis graph of partially purified bacteriocin reveals the presence of 3340.73 and 3201.83 cm^{-1} hydrogen bonded OH groups, 2866.87 cm^{-1} shows C-H streaching, 1689.64 shows carboxyl group, 1620 shows guasian amide bonds and 1504.48 shows carbon -carbon streaching. 894 shows $C\text{-}NH_2$ streaching. 4- Amino quinaldine or Na-acetate or C-C in plane. All above characteristics valley indicated peptide-based structure of the compound (Figure 4).



	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area	Comment
1	393.48	47.73	74.81	420.48	358.76	-361.931	1264.235	
2	659.66	80.68	15.34	709.80	570.93	1536.300	1025.736	
3	740.67	75.16	29.80	786.96	709.80	618.110	1189.194	
4	894.97	87.85	13.48	929.69	786.96	-280.986	792.111	
5	1045.42	86.25	14.56	1087.85	995.27	583.494	654.120	
6	1138.00	85.74	19.76	1180.44	1087.85	468.083	947.507	
7	1215.15	90.33	20.68	1257.59	1180.44	-82.126	784.351	
8	1292.31	88.96	27.13	1338.60	1257.59	-214.027	1124.276	
9	1504.48	60.18	28.98	1550.77	1473.62	1960.771	1198.066	
10	1620.21	31.74	62.28	1666.50	1550.77	4268.830	3594.033	
11	1689.64	81.42	24.38	1805.37	1666.50	-4729.308	-491.055	
12	2866.22	174.00	11.40	2920.23	2812.21	-8729.698	493.884	
13	3201.83	92.66	76.29	3255.84	3070.68	-7660.139	5113.699	
14	3340.71	85.20	114.68	3402.43	3255.84	-6785.265	7237.017	

Figure.4 FTIR analysis of partially purified bacteriocin.

IV Conclusions

The *Bacillus subtilis* NCIM 2548 culture fulfills most of the criteria which are used in the selection of probiotic bacteria. It was resistance to low pH. It could tolerate high temperature and 0.3% bile concentration. Production and extraction of bacteriocin were carried out successfully.

Antimicrobial activity of crude and partially purified bacteriocin against *S.aureus* shows maximum inhibition zones 18±0.63 mm and 21±0.85 mm respectively. The bacteriocin of *Bacillus subtilis* showed moderate antioxidant activity by exhibiting 62% inhibition of free radical. Ascorbic acid was used as standard control and it showed 87% inhibition of free radical. From FTIR analysis one may conclude that partially purified bacteriocin was peptide based compound.

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