Bacteriocin (BAC-IB17): Screening, isolation and production from *Bacillus subtilis* KIBGE IB-17

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Abstract: Bacteriocins are peptides produced by a variety of different microbes and have antimicrobial activity against closely related species. These antimicrobial agents are gaining more and more attention as an alternative therapeutics not only in pharmaceutical but also as a preservative in food industries. In this study several bacterial strains were isolated from soil and screened for bacteriocin production. Among them, one strain identified as *Bacillus subtilis* KIBGE IB-17 on the basis of taxonomic studies and confirmed by 16S rDNA analysis. This newly isolated strain showed antibacterial activity against several Gram positive and Gram negative bacteria. Different concentrations of tryptone, yeast extract and NaCl and physiochemical factors such as temperature, pH and incubation period were selected as variables for maximum production of bacteriocin by using agar well diffusion method and significant effects of variables were observed on the production of Bac-IB17. A newly designed modified TY medium showed maximum bacteriocin production containing 1.0% tryptone, 0.5% yeast extract and 0.5% NaCl. Maximum Bac-IB17 production was observed at 37ºC after 24hours with initial medium pH 7.0. *Bacillus subtilis* KIBGE IB-17 is capable of producing a bacteriocin at a wide range of pH and temperature that makes it an ideal strain that can be used for the production of bacteriocin on industrial scale level. The identification and production of such bacteriocin like compound against a wide spectrum of microbial species is very important for food and pharmaceutical industry.

Keywords: Bacteriocin, antimicrobial activity, *Bacillus subtilis*, agar well diffusion method.

INTRODUCTION

Production of antimicrobial substance is an important factor in microbial ecology. Many substance play a key role in bacterial interactions, among them bacteriocins are highly specific and efficient antagonist (Sahl, 1994). Bacteriocins are the peptides and protein antibiotics which are produced by several species and have antimicrobial properties usually against other closely related species (Cladera-Olivera, *et al.*, 2004). Several kinds of bacteriocins may be produced within same species and they are ribosomally synthesized in the host while the producer strain possesses specific self protection mechanism against this bacteriocin (Sahl, 1994; Motta and Brandelli, 2008). They are heterogeneous compounds having variability in biochemical properties, molecular weight, activity spectra and mode of action (Klaenahmmer, 1998). These antimicrobial peptides are gaining more and more attention not only as an alternative therapeutic agent for the prevention and treatment of infections but also as preservatives in food industries to avoid deterioration and spoilage of food (Anthony *et al.,* 2009). Bacteriocins are generally recognized as naturally occurring food preservatives able to influence the quality and safety of foods (Settanni and Corsetti, 2008). Non-clinically, bacteriocins also have applications to control animal and food borne pathogens in live stock (Diez-Gonzalez, 2007).

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Bacteriocins produced by Gram positive bacteria have been largely studied and also biochemically and genetically characterized (Navaratna, 1998). Bacteriocin activity is very specific and due to difference in cell wall composition, the activity spectra by Gram positive bacteria are wider as compare to Gram negative bacteria. Bacteriocins of Gram positive and Gram negative bacteria have evolved differently in terms of size and specificity (Jack, 1995)**.** Currently, bacteriocins produced from lactic acid bacteria are studied extensively due to their generally recognized as safe (GRAS) status (O'Sullivan, 2002). Most of the species from genus *Bacillus* are also considered as industrially important bacteriocin producers and have a history of safe use (Cladera-Olivera, *et al.*, 2004) and they are also GRAS microorganisms (Martirani *et al.*, 2002). Many bacteriocins and bacteriocin-like inhibitory substances have been classified for LAB, but still now limited classification scheme has been devised for *Bacillus* bacteriocins (Abriouel *et al.*, 2011). One of the most important specie of genus bacillus is *Bacillus subtilis* that is commonly recovered from water, soil and environment and it can survive in extreme conditions of heat and desiccation because of the production of endospore (Alexander, 1977). *B. subtilis* is considered a benign organism as it is non-pathogenic and non-toxigenic to humans, animals and plants and it does not possess any traits that cause disease (Erikson, 1976). Beside the production of various industrially important enzymes, *B. subtilis* has been shown to produce a wide variety of antibacterial and antifungal compounds (Katz and Demain, 1977; Korzybski *et al.*, 1978; Stein, 2005).

Antibiotic resistance of pathogenic bacteria is increasing day by day because of over-prescribing of antibiotics and inadequate use of drugs. This concern is becoming a challenge for researchers to investigate new antimicrobial agents produced by bacterial strains of apparent low virulence and having antibacterial activity against wide range of clinically significant organisms (Jack, 1995). Current study deals with the isolation, characterization and effect of culture conditions on the production of a new bacteriocin-like inhibitory substance designated as Bac-IB17 produced by *Bacillus subtilis* KIBGE IB-17 with promising properties as an antimicrobial agent against different pathogenic species.

MATERIALS AND METHODS

Organism

Bacillus subtilis KIBGE IB-17 was screened and isolated from soil sample obtained from a vegetative field in Karachi, Pakistan. Screening for bacteriocin production was performed using stab and overlay method. The culture was maintained on nutrient agar with pH 7.2 at 4ºC.

Taxonomic characterization of the isolate

Identification of the genus was based on morphological and biochemical characteristics for *Bacillus* species (Holt *et al.*, 1994). All the tests were performed on liquid cultures in late-logarithmic phase.

16S rDNA gene analysis and sequencing

A. Extraction of bacterial DNA

The DNA was extracted using the method as described by Chen and Kuo (1993) with slight modifications and visualized on 1.0% agarose gel.

The 16S ribosomal DNA (rDNA) was amplified by PCR using 16SF: GAGTTTGATCCTGGCTCAG and 16SR: AGAAAGGAGGTATCCAGCC as primers. Thermal cycling was performed in an Applied BioSystem GeneAmp PCR system 2700 using Taq Polymerase (Fermentas) according to the following program: initial denaturation at 94°C for 4 min; 35 cycles of 94°C for 1 min., 55°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 7 minutes.

The PCR product after visualization was purified from the gel using PCR product purifying kit of Wizard SV Gel and PCR Cleanup System (Promega). The sequencing was performed by the DNA Sequencer ABI 3130 Genetic Analyzer (Applied BioSystems). 16S rDNA sequence was compared to other prokaryotic 16S rDNA sequences by using the similarity rank analysis Service of NCBI (BLAST). For the construction of phylogenetic tree and determination of the nearest database neighboring sequences, the sequences of all the five isolates were aligned using CLUSTAL X program version 1.8 (Thompson *et al.,* 1997). Phylogenetic tree was constructed using the neighbor joining algorithm of CLUSTAL X and displayed by using Tree View (Page, 1996). The sequences for the closest neighbors (approx. bp 1600) were used for this purpose. A total of 40 sequences were aligned from the genus *Bacillus* sequences available at NCBI GenBank database.

Bacteriocin production

Bacteriocin production by *B. subtilis* KIBGE IB-17 was carried out in modified TY medium (Tryptone, 10.0; Yeast extract, 5.0; NaCl, 5.0 g-^{1}L) having initial pH 7.0 and was sterilized at 121ºC for 15 minutes. Inoculum (100ml) was grown in the medium at 37 ºC for 24 hours and was transferred into 900ml of fermentation medium and incubated at 37 ºC for 24 hours with an agitation of 135 rpm. Cells were harvested by centrifugation at 15000 rpm for 15 minutes at 4˚C and cell free supernatant was sterilized with 0.22µm filter membrane under sterile conditions and stored at -20˚C for further studies.

Antimicrobial activity assay

The antimicrobial activity of bacteriocin (cell free supernatant) was detected against different indicator strains listed in table 1 by agar well diffusion method (Tagg and McGiven, 1971). Cell free supernatant (100 µl) was added in 10 mm wells on nutrient agar plates previously spreaded with 100 µl suspension of each indicator strain containing $2x10^{8}$ cfu/ml (Iqbal, 1998). The plates were incubated for 24-48 hours at specific temperature according to indicator strains used (fig. 1). All values represented in table 1 are the average of three observations.

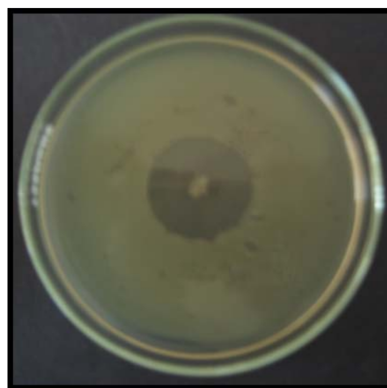

Fig. 1: *Bacillus subtilis* KIBGE IB-17 showing Anti-Bacterial Activity by Stab and Overlay Method against *Micrococcus* sp. (KIBGE IB-20) as Sensitive Culture.

Effect of different media on bacteriocin production

Three different media were used for bacteriocin production including nutrient broth, BHI broth and TY broth at 37˚C for 24 hours (Cladera-Olivera *et al.,* 2004; Motta and Brandelli, 2008; Martirani *et al.*, 2002). TY medium was modified for the maximum bacteriocin production by *B. subtilis* KIBGE IB-17 (fig. 2).

Table 1: Antimicrobial activity spectrum of Bac-IB17 produced by *Bacillus subtilis* KIBGE IB-17

Indicator strains	Source	Zone of inhibitions (mm)	Antagonistic activity
Micrococcus sp.	Soil (KIBGE IB-20)	30	++++
Staphylococcus aureus	Clinical Isolate	28	++++
Bacillus stearothermophilus	Clinical Isolate (KIBGE IB-18)	25	+++
Enterococcus faecalis	ATCC 29212	22	+++
Listeria monocytogenes	Food Sample	21	+++
Eschericia coli	Drinking Water	18	++
Salmonella typhi A	Clinical Isolate	15	+
Salmonella typhi B	Clinical Isolate	0	-
Salmonella typhi	Clinical Isolate	0	-
Salmonella typhimurium	ATCC 3632	0	-
Bacillus licheniformis	Environment (KIBGE IB-1)	0	-
Bacillus licheniformis	Soil (KIBGE IB-3)	0	-
Staphylococcus aureus	ATCC 6538	0	-
Pseudomonas aeruginosa	Drinking Water	0	-
Bacillus cereus	ATCC 11778	0	-

(-) No Antgonistic Activity; (+) Inhibitory zone diameter within 11-15; (++) Inhibitory zone diameter within 16-20; (+++) Inhibitory zone diameter within 21-25; (++++) Inhibitory zone diameter within 26-30.

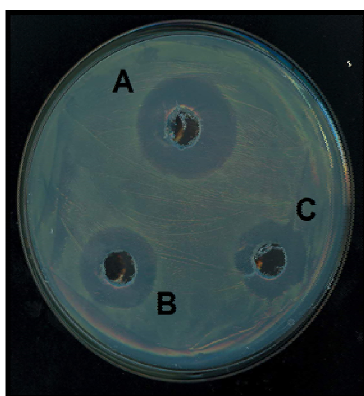

Fig. 2: Selection of medium for bacteriocin production by agar well diffusion method. **A**: Modified TY broth; **B**: BHI broth; **C**: Nutrient broth.

Effect of NaCl, tryptone and yeast extract concentration on bacteriocin production

Different concentrations of NaCl were used in the modified TY medium ranging from 0.2 to 1.5%. Similarly, tryptone and yeast extract concentrations were also varied from 0.1 to 2.5% and 0.25 to 2%, respectively. All the experiments were performed in triplicate and the results are the mean of the observations.

Effect of incubation time, temperature and pH on bacteriocin production

For maximum bacteriocin production the fermentation was carried out at different time intervals ranging from 06 to 120 hours. Optimum temperature for maximum bacteriocin production was achieved by incubating the culture medium at different temperatures from 20˚C to 60˚C for 24 hours. Similarly, pH of media was also varied ranging from 4 to 10 for the maximum bacteriocin production. All the experiments were performed in triplicate and the results are the mean of all the observations.

RESULTS

Effect of different media on bacteriocin production

Several reported medium compositions were used for bacteriocin production. Among them TY medium (Martirani *et al.*, 2002) with agitation was initially selected for bacteriocin production by KIBGE IB-17 but maximum bacteriocin production was attained when modified medium was used (table 2).

Table 2: Medium selection for optimum Bac-IB17 production by *Bacillus subtilis* KIBGE IB-17 against *Micrococcus* sp. KIBGE IB-20

Media	Fermentation with shaking	Fermentation without shaking
	Zone of inhibitions (mm)	Zone of inhibitions (mm)
Nutrient broth	23 ±3	20 ±2
BHI broth	25 ±3	23 ±3
TY broth	27 ±4	23 ±3
Modified TY broth	30 ±3	25 ±3

Effect of NaCl, tryptone and yeast extract concentration on bacteriocin production

When NaCl concentration was varied for bacteriocin production it was observed that at 0.5% maximum production was achieved and maximum zone of inhibition was observed. A decline was observed in Bac-IB17

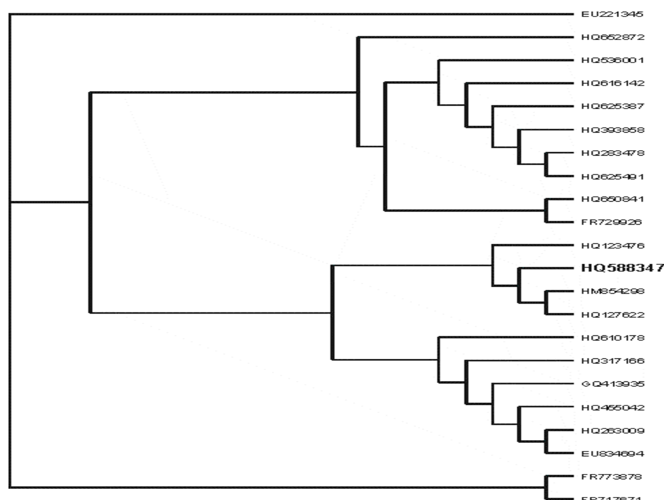

Fig. 3: Phylogenetic tree constructed based on 16S rDNA gene sequence analysis for *B. subtilis* KIBGE IB-17 with reference sequences available at NCBI through BLAST analysis. Sequence highlighted in this figure is from the current study.

production as the concentration the salt was increased (fig. 4). It was observed that as the concentration of tryptone increases from 0.1% to 1.0%, Bac-IB17 production gradually increased and after reaching maxima it started to decline (fig. 5). In case of yeast extract maximum Bac-IB17 production was observed at 0.5% concentration that followed with a sudden decline in activity as yeast extract concentration was raised (fig. 6).

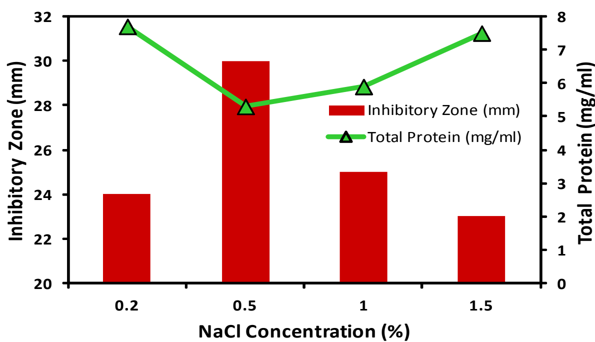

Fig. 4: Production of Bac-IB17 at different NaCl concentrations.

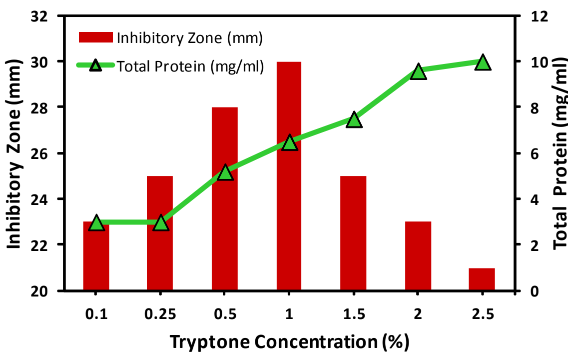

Fig. 5: Production of Bac-IB17 at different tryptone concentrations.

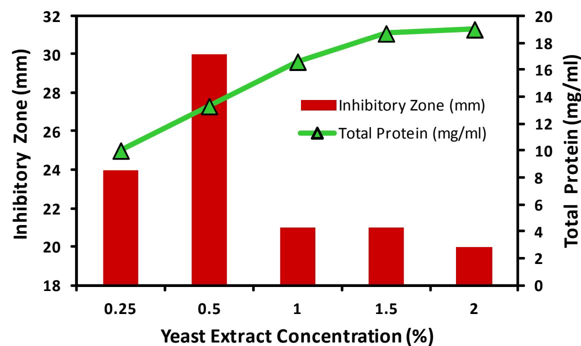

Fig. 6: Production of Bac-IB17 at different yeast extract concentrations.

Effect of incubation time, temperature and pH on bacteriocin production

It was observed that bacteriocin production started after 06 hours of incubation but maximum specific production rate was achieved after 24 hours and as time increases production decreases and after 72 hours no production was observed (fig. 7).

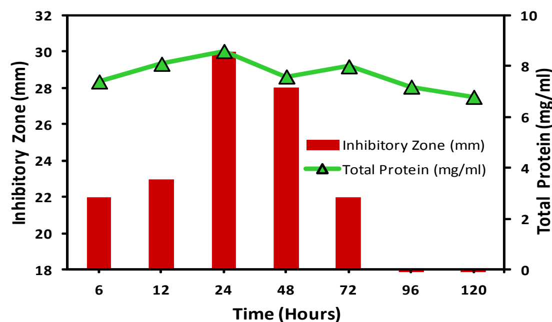

Fig. 7: Production of Bac-IB17 at different time intervals

Temperature play a key role on bacteriocin production and it was observed that maximum production was achieved at 37°C whereas, it was also produced even at 20°C and this might be first evidence that even at lower temperatures *Bacillus subtilis* is capable of producing bacteriocin (fig. 8). Maximum Bac-IB17 production was observed at pH 7.0 (fig. 9); however it was also observed that production was also achieved at slightly acidic condition (pH-6.0) and as the pH increases upto 9.0, production decreases but retained it activity.

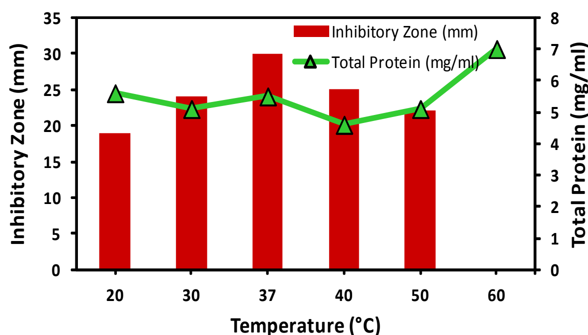

Fig. 8: Production of Bac-IB17 at various temperatures.

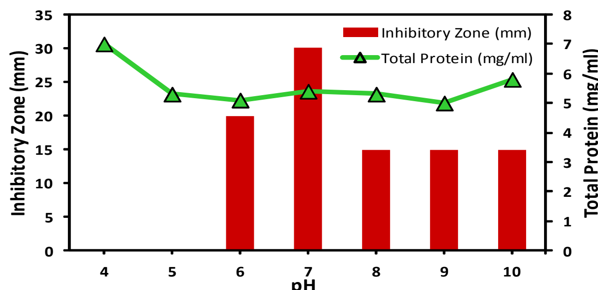

Fig. 9: Production of Bac-IB17 at different pH values.

DISCUSSION

Several strains of *Bacillus* species were isolated from soil of vegetative field and out of which one strain showed prominent antimicrobial activity. This producer strain was identified as *Bacillus subtilis* KIBGE IB-17 on the basis of morphological, physiological and biochemical characteristics and further confirmation was made by 16S rDNA cataloging (GenBank accession numbers from NCBI Nucleotide DatabaseHQ 588347). Bacterial isolates obtained in the current study showed a prokaryotic diversity analysis among the microbial community and belongs to the phylum *Firmicutes*. A 16S rDNA sequence for *B. subtilis* KIBGE IB-17 was used to generate the phylogenetic tree (fig. 3).

The antibacterial activity of Bac-IB17 was observed against Gram positive bacteria (Table 2). When cell free supernatant (Bac-IB-17) was tested against 15 indicator strains, 07 were found to be sensitive. Highest zone of inhibition was observed against *Micrococcus sp.* and clinical isolate of *Staphylococcus aureus.* When *Bacillus stearothermophilus*, *Enterococcus faecalis* and *Listeria monocytogenes* were used as indicator strains, moderate zone of inhibition were observed. In case of Gram negative bacteria zone of inhibition were observed only against *Escherichia coli* and *Salmonella typhi* A, however the inhibition was very low as compared to Gram positive. While, eight other bacterial species showed no inhibition and they were completely resistant to Bac-IB17.

Maximum bacteriocin production was achieved in modified medium with 0.5 % NaCl concentration. As the concentration of NaCl increases, production decreases (fig. 4). As bacteriocin are protein in nature, total protein concentration was also determined and medium containing NaCl (0.5%) showed maximum specific activity with reference to zone of inhibition.

Similarly after achieving maximum production at 1% tryptone, decline in Bac-IB17 production might be due to the inhibitory effect of this nitrogen source. Total protein concentration also increased as the concentration of tryptone increased but the specific activity with reference to zone of inhibition decreased in media containing tryptone ranging from 1.5 % to 2.5% as compared to the initial concentrations. It was reported by Ansen *et al.*, (2002) that increased concentration of yeast extract and tryptone in the medium increased the production rate of a bacteriocin Sakacin P. Similarly, in case of Bac-IB17 a modified medium having 1% tryptone, 0.5% yeast extract and 0.5% NaCl played a vital role in its production.

Incubation time plays a vital role in bacteriocin production and after 24 hours maximum specific production rate was achieved. It was observed that Bac-IB17 production started in initial 06 hours of incubation but maximum specific production rate was achieved because at this time the cells were in the exponential phase reaching towards stationary phase at 48 hours (data not shown). Antibacterial activity of Bac-IB17 then started to decline and after 72 hours, there was no zone of inhibition detected which showed that Bac-IB17 produced either lost its activity during incubation or become unstable after 72 hours (fig. 7). It was reported earlier that a bacteriocin was produced from *Bacillus subtilis*-14B after 96 hours of incubation (Hammami *et al.*, 2009).

Maximum production of Bac-IB17 was achieved at 37ºC and this bacterium is also capable of producing bacteriocin even at low temperature (fig. 8). This property of Bac-IB17 produced by *Bacillus subtilis* KIBGE IB-17 can be used as a preservative in food processing industries to avoid food spoilage even at cold temperatures. However, a lactic acid bacterium is also reported for

bacteriocin production at low temperatures (Ansen *et al.*, 2002). Bac-IB17 production was also detected at 50°C but no bacteriocin production was found at 60°C because there was no microbial growth observed as this temperature was inhibitory for bacterial growth of *B. subtilis* KIBGE IB-17. Bacteriocins produced in alkaline conditions are now gaining more attention in several food industries as pH of many food products is between neutral to alkaline. It is previously reported that nisin is the only bacteriocin used commercially as a food additive in acidic conditions while it is unstable at alkaline pH (Liu and Hansen, 1990). Another bacteriocin from *B. licheniformis* known as bacillocin 490 showed antibacterial activity between acidic to alkaline pH (Martirani *et al.*, 2002).

CONCLUSION

Present study is based on the screening, isolation and growth kinetics of Bac-IB17 production from *Bacillus subtilis* KIBGE IB17 with promising features for industrial applications. Bac-17 displayed antibacterial activity against Gram positive as well as Gram negative pathogens. Maximum Bac IB-17 production was observed after 24 hours at 37°C keeping the initial pH of the medium at 7.0 Results revealed that Bac-IB17 has a potential to be used as an alternative therapeutic agent in pharmaceutical products as well as preservative in food industries. For this purpose complete purification, characterization in terms of stability, influence of various physiochemical factors is further required.

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