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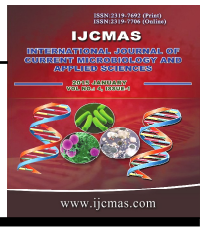

Original Research Article

Optimization of bacteriocin production by *Bacillus subtilis* BMP01 isolated from *Solanum trilobatum* L.

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Keywords

Bacteriocins, endophytic bacteria, *Solanum trilobatum*, optimization

A B S T R A C T

Bacteriocins are antibacterial proteins produced by bacteria that kill or inhibit the growth of other bacteria. In the present study, we investigated optimization of bacteriocin production by endophytic bacteria *Bacillus subtilis* BMP01 isolated from the medicinal plant *Solanum trilobatum* L. The isolated endophyte was identified based on the morphological and biochemical characteristics. The antibacterial activity of bacteriocins was evaluated against two indicator strains such as *Escherichia coli* and *Staphylococcus aureus*. The optimum incubation time, pH and temperature of bacteriocin production by *Bacillus subtilis* BMP01 was 24 hrs, 7 and 37ºC respectively. Results revealed that *Bacillus subtilis* BMP01 is a potential source for bacteriocin production.

Introduction

Bacterial antimicrobial peptides produced by ribosomal synthesis are commonly referred to as bacteriocins, and these are a heterologous group of proteinaceous antimicrobial substances that are produced by bacteria from every major lineage (Riley and Wertz, 2002a, b). They display a high degree of target specificity against related bacteria, although many have a wider spectrum of activity (Jack *et al.*, 1995).Their proteinaceous nature implies a putative degradation in the gastrointestinal tract of humans and animals, suggesting their use as natural preservatives in foods (Cleveland *et al.*, 2001).

The first bacteriocin colicin was produced by *Escherichia coli* (Cascales *et al.*, 2007). Though, currently, the bacteriocins most studied are those produced by lactic acid bacteria (LAB), because of their potential use as biopreservatives in the food industry, considering the 'generally recognized as safe' (GRAS) status of many strains (O'Sullivan *et al.*, 2002).

Members of the genus *Bacillus* are known to produce a wide arsenal of antimicrobial substances, including peptide and lipopeptide antibiotics, and bacteriocins. Many of the *Bacillus* bacteriocins belong to the lantibiotics, a category of post-translationally modified peptides widely disseminated among different bacterial clades. *Bacillus* bacteriocins are increasingly becoming more important due to their sometimes broader spectra of inhibition (as compared with most LAB bacteriocins), which may include Gram-negative bacteria, yeasts or fungi, in addition to Gram-positive species, some of which are known to be pathogenic to humans and animals.

Bacteriocins produced by industrially important *Bacillus subtilis*, which have a history of safe use in food and industry (Pedersen *et al.,* 2002). *Bacillus subtilis* strains are used in Natto, an East Asian fermented food (Hosoi and Kiuchi, 2003), production. *B. subtilis* strains are also used as a starter culture for fermenting soybeans into the traditional West African condiment dawadawa (Terlabie *et al.,* 2006) or for fermenting African mesquite seeds in the production of the Nigerian food condiment okpehe (Oguntoyinbo *et al.,* 2007). Consequently, in the present investigation was deliberated to evaluate the optimization of bacteriocin production by endophytic bacteria *Bacillus subtilis* BMP01.

Materials and methods

Isolation and identification of bacterial strain

The bacteriocin producing strain was isolated from leaves of *Solanum trilobatum* L., the selected strain was identified based on its morphological and biochemical characteristics.

Bacteriocin production (Ansari *et al.,* 2012)

Bacteriocin production by *Bacillus subtilis* BMP01 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^{o}C for 15 minutes. Inoculum (100ml) was grown in the medium at 37^{o}C for 12, 24, 36, 48, 60 and 72 hours respectively. One of the samples was used for measuring optical density (O.D.) at 600 nm. After each respective incubation period, the broth were transferred to 500 ml conical flask and placed in shaking incubator with the agitation of 150 rpm for 10 minutes. After agitation, the cells were harvested by centrifugation at 10000 rpm for 10 minutes at 4^{o}C and cell free supernatant was filtered through 0.22µm filter membrane under sterile conditions and stored at -20^{o}C for further studies.

Antibacterial assay

The antibacterial activity of bacteriocin (cell free supernatant) was detected against *Escherichia coli* and *Staphylococcus aureus* by agar well diffusion method (Tagg and McGiven, 1971). Cell free supernatants (25µl) from different incubation period (12, 24, 36, 48, 60 and 72 hours) were added to 5 mm wells on nutrient agar plates. Nutrient agar plates were spread with 100 µl suspension of each indicator strain containing 2 x 10^{8} cfu/ml (Iqbal, 1998). The plates were incubated for 24 hours at specific temperature according to indicator strains used. All the experiments were performed in triplicate and the results are the mean of the observations. The antagonistic activity in arbitrary unit/ml (AU/ml) was calculated (Bhaskar *et al.,* 2007) as a measure of bacteriocin production.

$$AU/ml = \frac{\text{Diameter of the zone of clearance (mm)} \times 1000}{\text{Volume taken in the well (µl)}}$$

Effect of pH and temperature

To analyze thermal stability, aliquots of produced bacteriocins were exposed to different temperature (27°C, 37°C, 47°C, 57°C and 67°C) for 15 minutes and effects of pH on activity of bacteriocin were determined by adjusting the pH of the produced bacteriocin with diluted HCl and NaOH. The samples were incubated at different pH (pH - 3, 5, 7, 9 and 11) for 24 hour at 37°C and tested for antibacterial activity against the selected indicator strains.

Results and Discussion

Members of the genus *Bacillus* are Gram-positive, aerobic and endospore forming bacteria that are characterized by their rod shaped cell morphology, catalase production and their ubiquitous distribution. They are found in diverse environments such as soil and clays, rocks, dust, aquatic environments, plants, food and the gastrointestinal tracts of various insects and animals (Nicholson, 2002).

In the present investigation, *Bacillus subtilis* BMP01 was isolated from leaves of *Solanum trilobatum* L., identified based on its morphological and biochemical characteristics. The results were given in table 1. The antibacterial activity of bacteriocins was observed against two indicator strains such as *Escherichia coli* and *Staphylococcus aureus.* Highest zone of inhibition was observed against *Staphylococcus aureus.* Previously, *Bacillus subtilis* LFB112 from Chinese herbs produces a bacteriocin against both gram-positive and gram-negative bacteria involved in domestic animal diseases, including *E. coli, Salmonella pullorum, P. aeruginosa, Pasteurella multocida, Clostridium perfringens, Micrococcus luteus, Streptococcus bovis* and *S. aureus* (Xie *et al.,* 2009). *Bacillus subtilis* 14B also isolated from the rhizosphere of healthy plants (bitter almond) produces a bacteriocin (Bac 14B) active against *Agrobacterium tumefaciens* (Hammami *et al.,* 2009).

Effect of incubation time on bacteriocin production

Incubation time plays a vital role in bacteriocin production and different incubation time was used in the modified TY medium from 12 to 72 hrs. The maximum bacteriocin production was achieved at 24 hours and as time increases production decreases (Table 2; Fig. 1 and 2; Plate 1). Correspondingly, Hammami *et al.* (2009) and Ansari *et al.* (2012) reported that the optimum incubation time of bacteriocin production from *Bacillus subtilis* was 24 hrs.

Effect of pH on bacteriocin production

Maximum bacteriocin production was observed at pH 7.0 (Table 3; Fig. 3 and 4; Plate 2); however it was also observed that production was also achieved at slightly acidic condition (pH-5.0) and as the pH increases up to 11.0, production decreases but retained it activity. Evidently, maximum Bac-IB17 production from *Bacillus subtilis* KIBGE IB-17 was achieved at pH 7.0 by Ansari *et al.* (2012). Bacteriocins from *B. licheniformis* showed antibacterial activity between acidic to alkaline condition (Martirani *et al.,* 2002).

Effect of temperature on bacteriocin production

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 27ºC and this might be evidence that even at lower temperatures *Bacillus subtilis* is capable of producing bacteriocin (Table 4; Fig. 5 and 6; Plate 3). This property of bacteriocins produced by *Bacillus subtilis* BMP01 can be used as a preservative in food processing industries to avoid food spoilage even at cold temperatures. Bacteriocin production was also detected at 57ºC but no bacteriocin production was found at 67^{o}C because there was no microbial growth observed as this temperature was inhibitory for bacterial growth of *Bacillus subtilis* BMP01. Similarly, Ansari *et al.* (2012) also reported no bacteriocin production was found at 60ºC.

Bacteriocins from *Bacillus* species offer a much broader spectrum of potential applications compared with LAB bacteriocins. In the present study, bacteriocins by *Bacillus subtilis* BMP01 exhibited antibacterial activity against gram positive as well as gram negative bacteria. Maximum bacteriocin production was observed after 24 hours at 37^{o}C keeping the initial pH of the medium at 7.0. Results revealed that *Bacillus subtilis* BMP01 is a potential source for bacteriocins production and bacteriocins could be used as an alternative therapeutic agent in pharmaceutical products as well as preservative in food industries.

Table.1 Morphological and biochemical characteristic of isolated endophyte

S. No.	Gram staining	Motility	Indole test	MR test	VP test	Catalase test	Oxidase test	Citrate test	Nitrate reduction test	TSI test	Urease test	Carbohydrate fermentation test
1.	+ve rod	Motile	-ve	-ve	+ve	+ve	-ve	+ve	+ve	Gas production & acid butt	-ve	+ve

Table.2 OD values (600 nm) at different incubation periods

Incubation Time (hrs)	OD values at 600 nm
12	0.51
24	1.29
36	1.01
48	0.99
60	0.85
72	0.49

Table.3 OD values (600 nm) at different pH

Different pH	OD values at 600 nm
3	0.95
5	1.31
7	1.52
9	0.83
11	0.59

Table.4 OD values (600 nm) at different temperature

Different Temperature (ºC)	OD values at 600 nm
27	1.25
37	1.36
47	0.86
57	0.53
67	0.27

Fig.1 Production of bacteriocin at different incubation time (Indicator strain - *Escherichia coli*)

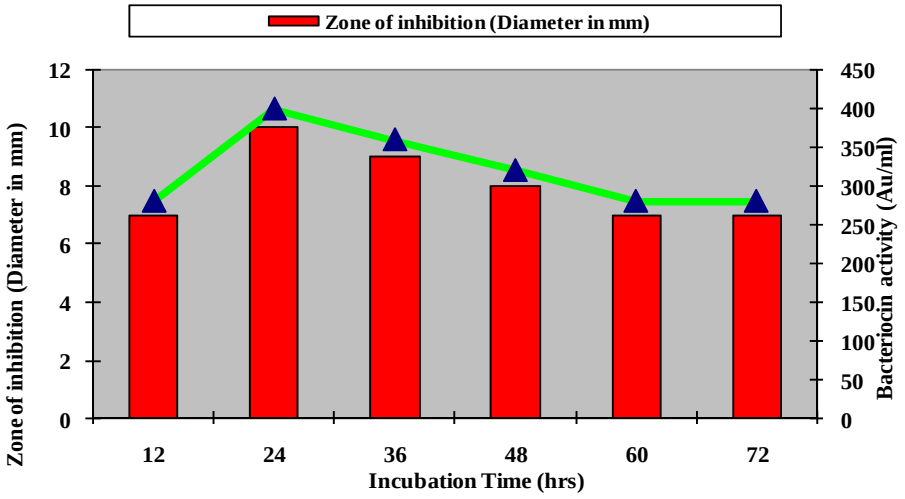


Fig.2 Production of bacteriocin at different incubation time (Indicator strain - *Staphylococcus aureus*)

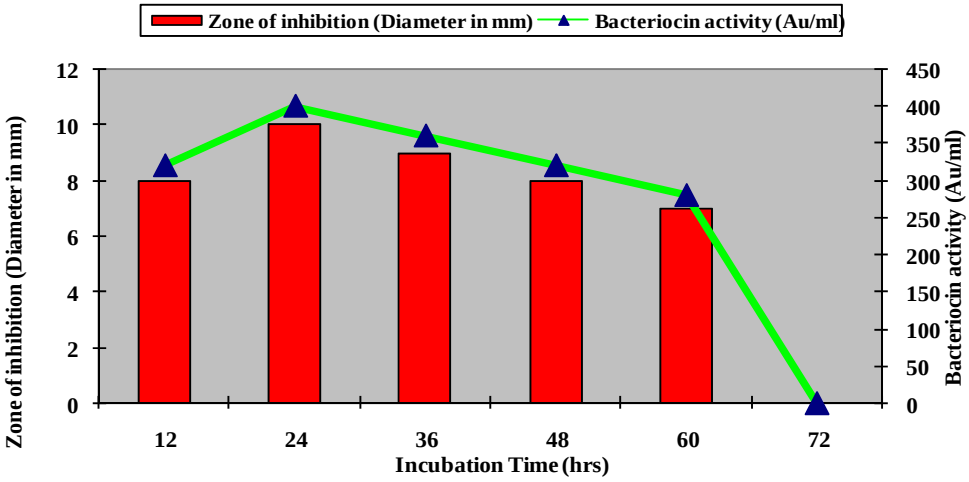


Fig.3 Production of bacteriocin at different pH (Indicator strain - *Escherichia coli*)

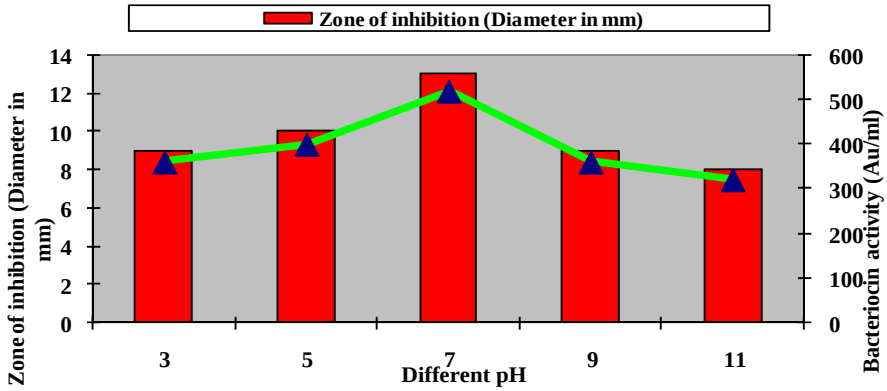


Fig.4 Production of bacteriocin at different pH (Indicator strain - *Staphylococcus aureus*)

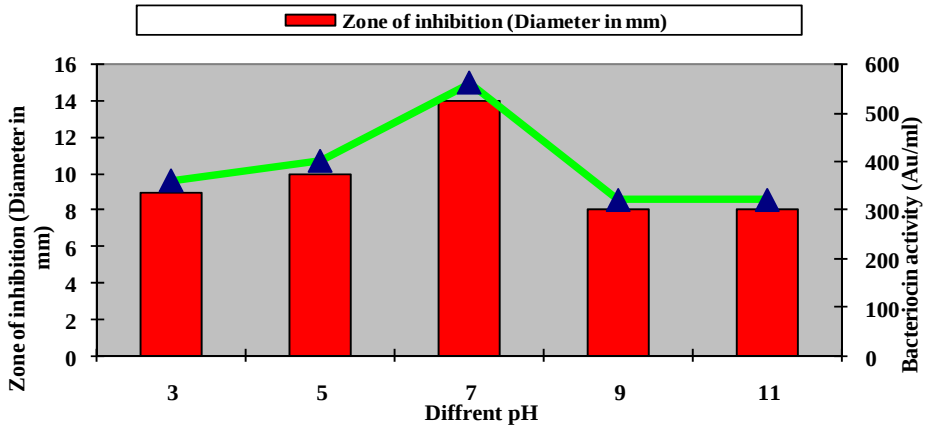


Fig.5 Production of bacteriocin at different temperature (ºC) (Indicator strain - *Escherichia coli*)

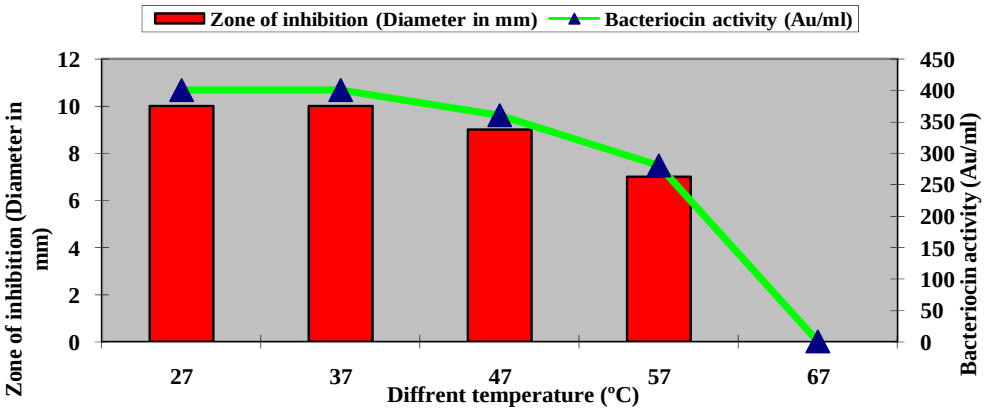


Plate.1 Effect of incubation periods on bacteriocin production by *Bacillus subtilis* BMP01

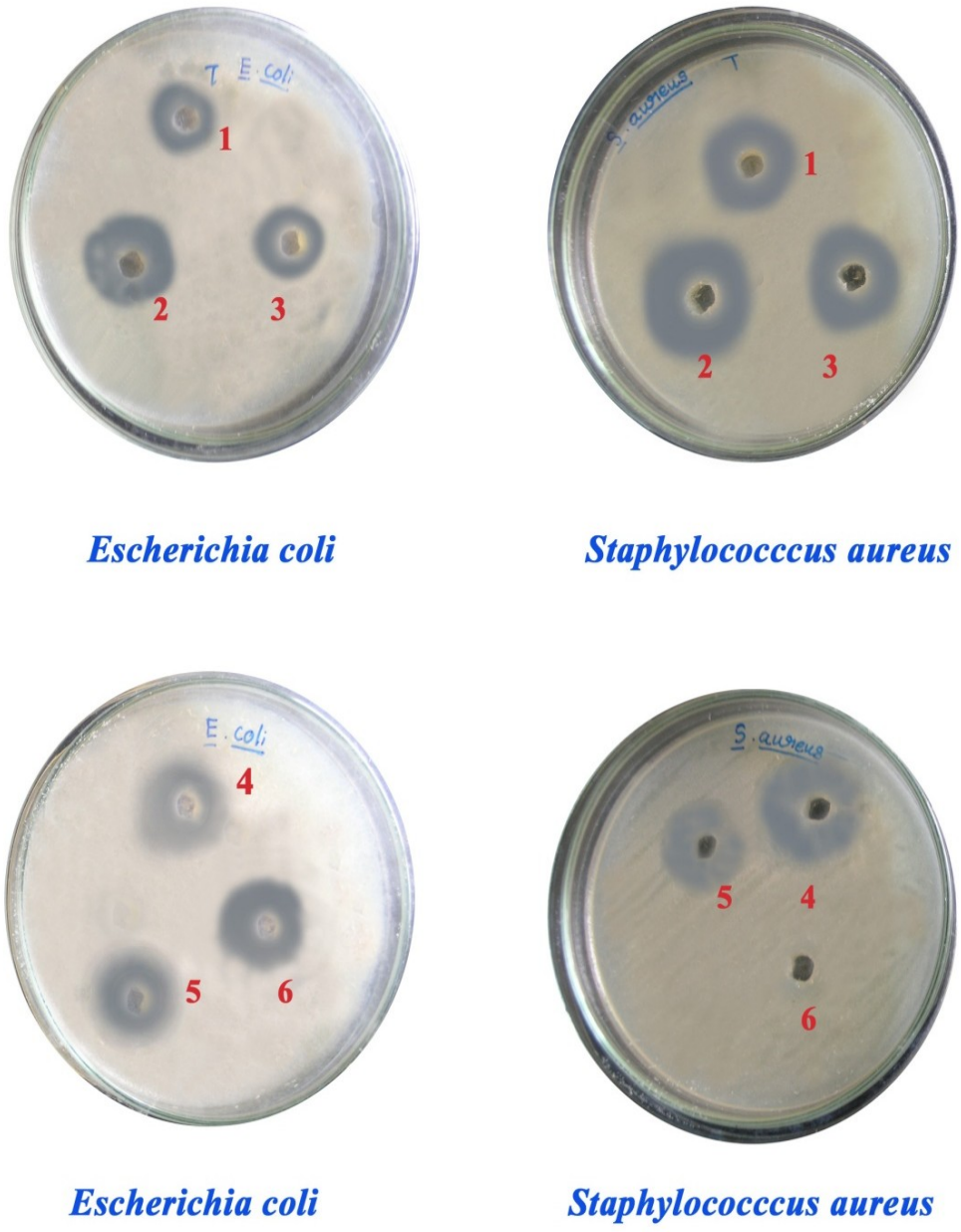
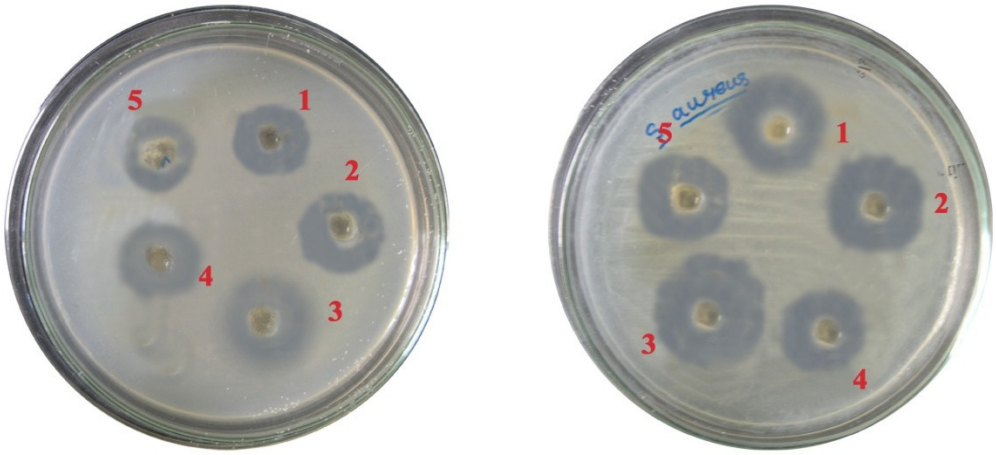


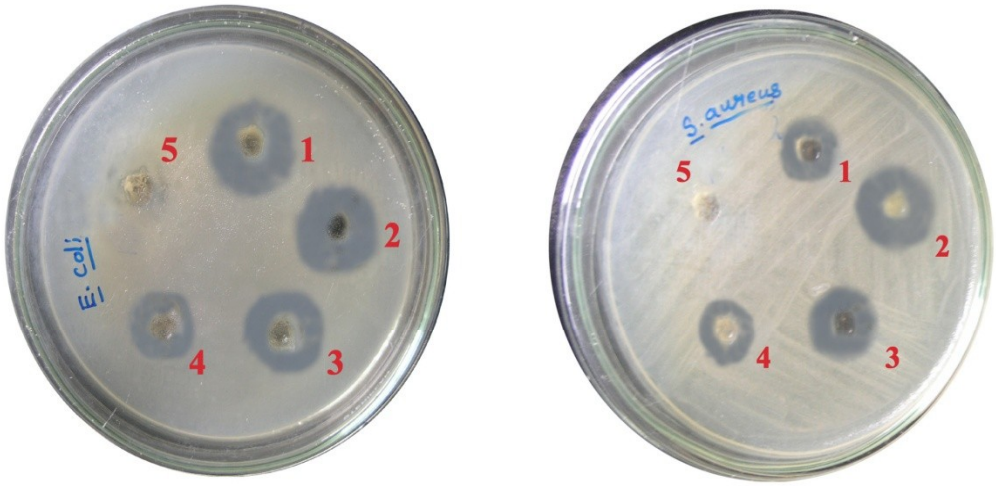
Plate.2 Effect of pH on bacteriocin production by *Bacillus subtilis* BMP01



Escherichia coli ***Staphylococccus aureus***

1 - pH 3, 2 - pH 5, 3 - pH 7, 4- pH 9, 5 - pH 11

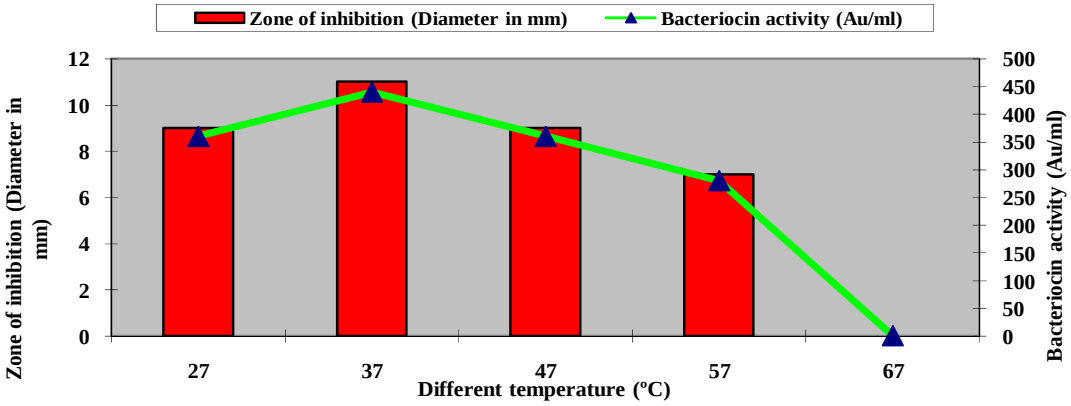
Plate.2 Effect of temperature on bacteriocin production by *Bacillus subtilis* BMP01



Escherichia coli ***Staphylococccus aureus***

1 - 27°C, 2 - 37°C, 3 - 47°C, 4 - 57°C, 5 - 67°C

Fig.6 Production of bacteriocin at different temperature (ºC)
(Indicator strain - *Staphylococcus aureus*)



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