

Studies on production of peptide antibiotic by thermotolerant *Bacillus sp.*

¹Joshi R. D., ²Hamde V.S., ³Umrikar A.M., ⁴Kulkarni S.S. and ⁵Bhate M.A

^{1, 3, 4, 5}Dept. of Microbiology, Shri Shivaji College, Parbhani (M.S.), India

²Yogeshwari Mahavidyalaya, Ambajogai. (MS). India.

Abstract

The soil sample was collected and screened for isolation of higher antibiotic producing bacterial strain. The isolated bacterial strain was tentatively identified as *Bacillus sp.* by biochemical characters using Bergey's manual. The present study is focused on the optimization of different parameters for production of peptide antibiotic (Bacitracin) using synthetic medium. The antibacterial activity of isolate was analyzed against *S. aureus*. The nutritional ingredients for bacitracin production were found as glucose (1%) and L-glutamic acid (0.5 %) as carbon and nitrogen source respectively. The prominent bacitracin production was obtained after 48 hrs incubation at 40°C temperature and at 7 pH.

Keywords: Biochemical characters ,peptide antibiotic, *Bacillus sp.*

INTRODUCTION

Screening of different antibiotics from natural sources is increasingly essential for the pharmaceutical industry as pathogenic bacteria develops resistance against commonly used therapeutic agents. Antibiotics are secondary metabolites produced by microorganisms in stationary phase of growth cycle, having diverse group chemical structure & biological activities produced specifically by *Bacillus* species [1]. Bacitracin is most important polypeptide antibiotic synthesized by *Bacillus* species, functions as a cell wall synthesis inhibitor. It is one of the important antibiotics used clinically in combination with other antimicrobial drugs [2].

These polypeptides are pharmacologically active secondary metabolites mainly recognized as only means of effective microbial growth control [3]. The majority of antibacterial substances from *Bacillus* species are low molecular weight substances produced via non-ribosomal biosynthetic pathway, which involves specific enzymes as peptide synthetases [4]. Peptide antibiotics are differing in their biochemical properties & play an essential role in non-specific host defenses by preventing the growth of potential pathogens [5]. Bacitracins are main antibacterial compounds produced by *Bacillus* species inhibiting the growth of gram positive bacteria, by blocking the cell wall synthesis & altering the cell membrane permeability. Different types of bacitracin like A, A₁, B, C, D, E, F, F₁, F₂ & G have been isolated. Bacitracin A is most potent, B & C are less potent & remaining are with very less antibacterial properties [6]. The empirical formula of bacitracin A is C₆₃H₁₀₃O₁₆N₁₇S. The different types of bacitracins are differing in amino acid residues. In alkaline condition the conformational structure of bacitracin may get change [7].

MATERIALS AND METHODS

Collection of samples and screening

The soil samples were collected and prepared serial dilutions from 10⁻¹ to 10⁻¹⁰. Dilutions were poured on antibiotic assay medium containing the test organism *Staphylococcus aureus*. All the plates were incubated at 37°C for 24 hrs. The plates were observed for appearance of zone of inhibition around the colonies. The higher antibiotic producing bacterial strain was isolated and identified by Bergey's manual.

Bacitracin production by *Bacillus sp* Inoculum preparation

The isolated bacterial strain was inoculated in nutrient broth and incubated at 37°C for 72 hrs having 150 rpm.

Production media

The 10% inoculum was added in bacitracin production medium contains (g/l): L-glutamic acid 5, KH₂PO₄ 0.5, K₂HPO₄ 0.5, MgSO₄ 0.2, MnSO₄ 0.01, NaCl 0.01, FeSO₄ 0.01, CuSO₄ 0.01, CaCl₂ 0.015, glucose 10, pH 7. The flask was incubated at 37°C in an orbital shaker adjusted at 150 rpm. The cells were harvested by centrifugation (3000 g for 10 min) after every 24 hrs to 144 hrs and collect supernatant for further study.

Agar diffusion assay

Agar well diffusion method was used to check the cultures for the production of antimicrobial metabolites [8]. The active culture of *Staphylococcus aureus* was diluted with pre sterilized saline. 0.1 ml culture of test organism was inoculated in nutrient agar plates by pour plate technique. By using borer, wells were prepared over nutrient agar plates. About 80 µl cell free supernatant was added in the wells. The plates were kept in refrigerator for 20 minute for diffusion of culture supernatant into the agar and incubated at 37°C for 24 hours. After 24 hours, the observed zones of inhibition were measured in terms of units of bacitracin.

Received: April 03, 2012; Revised: May 07, 2012; Accepted: June 02, 2012.

*Corresponding Author

Joshi R. D.
Dept. of Microbiology, Shri Shivaji College, Parbhani (M.S.), India

Email: purvarenuka@rediffmail.com

Effect of temperature and pH on antibiotic production

The effect of temperature on the antibiotic production was determined by performing the standard assay procedure at pH 7.0 within a temperature range from 30 to 50°C. Hydrogen ion activity was analyzed for bacitracin production by altering the pH from 6 to 9 of the medium using various buffers such as 50 mM acetate buffer, phosphate buffer, tris-HCl buffer and glycine-NaOH buffer [9].

Effect of C and N source on antibiotic production

The various types of carbons sources were applied on bacitracin production such as glucose, fructose, maltose, sucrose and manitol having 1 % concentration in production medium. As well as 1%, ammonium chloride, L-alanine and L-glutamic acid were used as a nitrogen source [10].

Effect of Incubation period on antibiotic production

The bacitracin production was measured after each successive 24 hrs, up to 96 hrs [11].

RESULTS

Identification of *Bacillus* species

The isolated species was identified on the basis of their morphological and biochemical characteristics [12]. The obtained data is mentioned in table 1.

Table 1. Biochemical characteristics of isolated *Bacillus spp.*

Test	Results
Catalase production	Positive
Oxidase	Positive
Citrate utilization	Positive
Methyl red	Negative
Indole production	Negative
Voges Proskauer	Positive
Nitrate reduction	Positive
Casein hydrolysis	Positive
Starch hydrolysis	Positive
Glucose fermentation	Negative

Identification of Bacitracin producing bacterial strain

The isolated species was tentatively identified on the basis of morphological & biochemical characters using Bergeys manual of determinative bacteriology. The bacterial isolate was Gram positive rods, spore forming & motile in nature. The isolated species was confirmed as *Bacillus* species. Sample was drawn during batch fermentation and analyzed it by agar diffusion assay, using *Staphylococcus* as test organism. Antimicrobial activity was measured in terms of zone of inhibition (mm).

Optimization of various parameters for maximum antibiotic production

The present investigation aimed to optimize the medium component that plays a significant role to enhance bacitracin production. The various parameters were tested against the bacitracin production such as incubation time, pH, temperature, carbon & nitrogen sources against the isolated bacterial strain. The

maximum activity was observed at pH 7. According to Yousaf (1997) [13], the production of bacitracin a enzyme was required as bacitracin synthetase. At lower and elevated pH the activity of the enzyme gets inhibited. The obtained results showed that, at pH 7 the units of bacitracin was 416 while at pH 5 and 9 units of bacitracin were 208 and 195 respectively. It indicates that, there was little antibacterial activity observed at acidic and alkaline conditions in favors the bacitracin production.

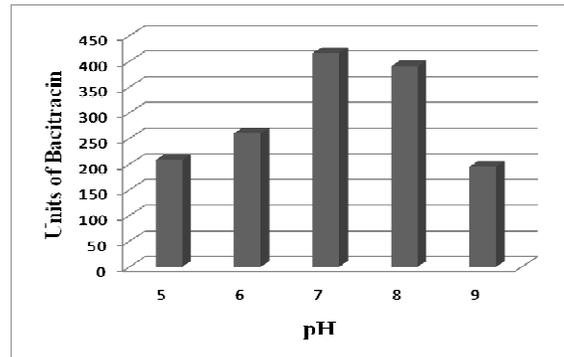


Fig 1. Antimicrobial effect of *Bacillus spp.* against *S.aureus* in terms of Units of Bacitracin at various pH.

Effect of incubation period on bacitracin production

The bacitracin production mainly observed at the end of logarithmic growth phase i.e. at start of stationary phase. It showed that, antibiotic production increases along with increase in incubation period up to arrival of stationary phase [3]. The isolated bacteria strain was having potential to produce elevated antimicrobial substances at 48 hrs and observed 520 units on bacitracin. At initial phase the isolated *Bacillus sp.* was unable to produce bacitracin where as at 96 hrs the bacitracin unit was 312.

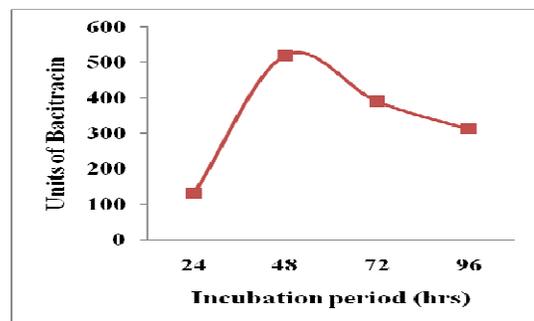


Fig 2. Antimicrobial effect of *Bacillus spp.* against *S.aureus* in terms of Units of Bacitracin at different time intervals

Effect of different carbon sources on bacitracin production

In the optimization of cultural conditions there was use of different carbon sources like glycerol, glucose, maltose, mannitol & fructose. Glucose was observed to be the best carbon source & gives maximum antibiotic production. Along with glucose glycerol also shows better antibiotic production as compare to other carbon sources. Glucose which is commonly a best carbon source may show inhibitory effect due to decreasing ph of the medium. Haavik (1974) [9] reported production of bacitracin by *Bacillus subtilis* is pH

dependent and inhibitory effect of glucose resulted due to accumulation of organic acids.

Effect of different nitrogen sources on bacitracin production

Nitrogen source was also the major components of the medium which influence the secondary metabolite production i.e. peptide antibiotics [14]. Bacitracin production was also affected by nitrogen sources. In this study L- glutamic acid & alanine acts as an enhancer of bacitracin production. Ammonium chloride as a nitrogen source not shows antibiotic production.

Effect of temperature on bacitracin production

The antibiotic i.e. production at different temperatures was depending upon organism selected for bacitracin production. The *Bacillus* species was thermo tolerant due to which they will also produce bacitracin at higher temperature like 50°C. At the time of consideration of antimicrobial activity, there was maximum production of bacitracin at 40°C there was also production of bacitracin at 50°C but activity was lowered down. At 60°C there was growth of organism but the antimicrobial activity was not determined. The effect of temperature was observed due to influence of it on growth rate of organism and velocity of enzymatic reactions.

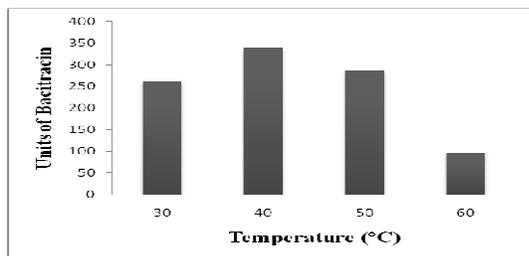


Fig 3. Antimicrobial effect of *Bacillus* spp. against *S.aureus* in terms of Units of Bacitracin at different temperatures.

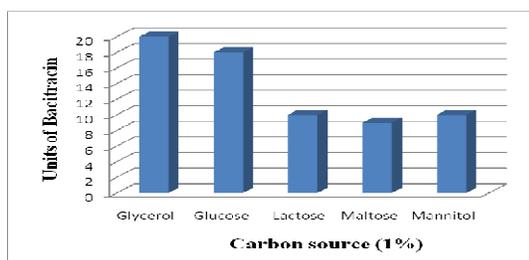


Fig 4. Antimicrobial effect of *Bacillus* spp. against *S.aureus* in terms of Units of Bacitracin at different carbon sources

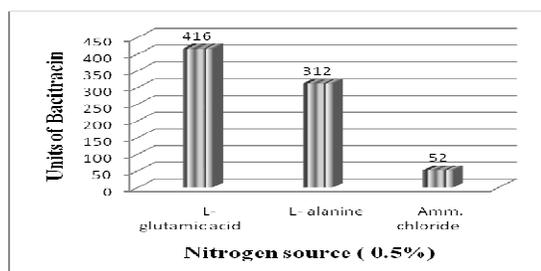


Fig 5. Antimicrobial effect of *Bacillus* spp. against *S.aureus* in terms of Units of Bacitracin at different Nitrogen sources

CONCLUSION

At the conclusive remark, it was observed that the isolated *Bacillus* species showed antibacterial activity against only gram-positive bacteria i.e. *S. aureus*. Bacitracin may be bactericidal or bacteriostatic in action. It inhibits bacterial cell wall synthesis by preventing incorporation of amino acids and nucleotides into the peptidoglycan layer. Production of bacitracin by *Bacillus* species was affected by many physicochemical factors.

REFERENCES

- [1] Azevedo, E.C.1993. Bacitracin production by a new strain of *Bacillus Subtilis*, extraction, purification and characterization. *Appl. Biochem. Biotechnol.*42: 1-7.
- [2] Bushra Jamil, Farina Hasan, A. Hameed and Safia Ahmed. 2007. Isolation of *Bacillus subtilis* MH-4 from soil and its potential of polypeptide antibiotic production. *Pak. J. Sci.* 20:26-31.
- [3] Aslim, B. Saglam, N. and Beyatli, Y. 2002. Determination of some properties of *Bacillus* isolated from soil. *Turk. J. Biol.* 26:41-48.
- [4] Eppelmann, K., Doekel, S. and Marahiel, M.A. 2001. Engineered biosynthesis of the peptide antibiotic bacitracin in the surrogate host *Bacillus subtilis*. *The J. Biol. Chem.*, 276:34824-34831.
- [5] Fariha Hasan, Samiullah Khan, Aamer Ali Shah and Abdul Hameed. 2009. Production of antibacterial compounds by free and immobilized *Bacillus pumilus* saf1. *Pak. J. Bot.* 41: 1499-1510.
- [6] Kalsoom Farzana, Syed Nisar Hussain Shah, Farooq Bashir Butt and Sattar Bukhsh Awan. 2005. Biosynthesis of bacitracin in solid-state fermentation by *Bacillus licheniformis* using defatted oil seed cakes as substrate. *Pakistan Journal of Pharmaceutical Sciences.* 55-57.
- [7] Kannikar Santong and Sumeth Naorungrote. 2008. Screening, Identification of antibacterial activities of effective thermotolerant *Bacillus* species isolated from raw milk. *Walailak J. Sci & Tech.* 5: 39- 46.
- [8] Mendo, S., Faustino, N.A., Sarmiento, A.C., Amado, F. and Moir, A.J. 2004. Purification and characterization of a new peptide antibiotic produced by a thermotolerant *Bacillus licheniformis* strain. *Biotechnol. Lett.* 26: 115.
- [9] Haavik, H. I.1974. Studies on the formation of bacitracin by *Bacillus licheniformis*: Role of catabolite repression and organic acids. *J. Gen. Microbiol.*84: 321.
- [10] Aftab, M. N., Haq, I. U. and Baig, S. 2010. Optimization of different parameters for the production of bacitracin in synthetic medium by *Bacillus licheniformis* mutant strain UV-MN-HN-8. *Pakistan Journal of Science.* 62:
- [11] Muhammad Awais, Aamer Ali Shah, Abdul Hameed and Fariha Hasan.2007. Isolation, Identification and Optimization of bacitracin produced by *Bacillus* sp. *Pak. J. Bot.*39: 1303-1312.
- [12] Buchanan, J. R. and Gibbons, N.E.1974. *Bergey's Manual of Determinative Bacteriology*, 8th ed., The Williams and Wilkins Company, Baltimore.
- [13] Yousaf, M.1997. Studies on the cultural conditions for the production of antibiotic bacitracin by *B.licheniformis*, Ph.D.

Thesis, Islamia University, Bahawalpur.

[14] Hanlon, G.W. and Hodges, N.A. 1981. Bacitracin and protease production in relation to sporulation during exponential growth

of *Bacillus licheniformis* on poorly utilized carbon and nitrogen sources. *J. Bacteriol.*147:427-431.