REPORT

ANTIBIOTIC PRODUCTION BY THERMOPHILIC *BACILLUS SPECIE* SAT-4

SYED AUN MUHAMMAD, SAFIA AHMAD* AND ABDUL HAMEED

Microbiology Research Laboratory, Department of Microbiology,
Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan

ABSTRACT

Production of antimicrobial compounds seems to be a general phenomenon for most bacteria. The prevalence of antimicrobial resistance among key microbial pathogens is increasing at an alarming rate worldwide. Current solutions involve development of a more rationale approach to antibiotic use and discover of new antimicrobials. *Bacillus* species produce a large number of biological compounds active against bacteria, fungi, protozoa and viruses. The process of production usually involves screening of wide range of microorganisms, testing and modification. Production is carried out using fermentation.

Thermophilic spore-forming, gram positive, motile rod bacterial strains were isolated from the Thar Desserts, Sindh Province, Pakistan. These strains were screened and checked for antibacterial activity. The best activity was observed by SAT4 against *Micrococcus luteus, Staphylococcus aureus and Pseudomonas aeroginosa*. The activity was only observed against gram positive bacteria and no activity was seen against *Pseudomonas aeroginosa.* Thermophilic *Bacillus specie* SAT4 was found to be active in the fermentation process to produce the antimicrobial agents. Further optimizations of different conditions (time of incubation, media, pH, glucose concentrations, nitrogen concentrations, and temperature) for antimicrobial production by the selected bacterial strain was performed. Agar diffusion assay was performed to evaluate the antibacterial activity. Optimum conditions for the production of antimicrobials by selected isolate were observed to be 48 hour, pH 5, temperature 55°C, 2% glucose and 1.5% nitrogen concentration. This newly isolated bacterial strain has great potential for antimicrobial production at industrial scale.

Keywords: Thermophiles, *Bacillus species*, antibiotics production.

INTRODUCTION

Antibiotics, in one form or another, have been in use for centuries. The vast majority of novel antibiotics have been detected by screening of "wild isolates" obtained from soil and other natural habitats. Although a wide taxonomic range of microbes have the ability to produce antibiotics, it would seem to be by no means universal. Thus, over 55% of the antibiotics detected between 1945 and 1978 originated from the genus *Streptomyces*, representing a total of more than 5,000 compounds (Berdy, 1980). With advances in organic chemistry many antibiotics are now also obtained by chemical synthesis, such as the sulfa drugs. Drugs used in the chemotherapy of infectious diseases are classified into two groups. Drugs that have been synthesized by chemical procedures in the laboratory are called synthetic drugs while those produced by bacteria and fungi are called antibiotics (Totora *et al*., 1995). The antibiotics are widely distributed in the nature, where they play an important role in regulating the microbial population of soil, water, sewage, and compost. Of the several hundred naturally produced antibiotics that have been purified, only a few have been sufficiently non-toxic to be of use in medical practice. Those that are currently of greatest use have derived from a relatively small group of microorganisms belonging to the genera *Penicillium*, *Streptomyces*, *Cephalosporium*, *Micomonospora* and *Bacillus* (Zinsser *et al*., 1988). Antibiotics, as secondary metabolites, are generally produced by multi-step biosynthetic pathways starting from intermediates of primary metabolism to specific moieties. Biosynthetic steps are catalyzed, by specific enzymes for each antibiotic (Demain *et al.*, 1983). Selective toxicity is possible because of occasional differences in the biochemistry of the host and microbial cell. For example, Penicillin inhibits the synthesis of bacterial cell wall, a component that is not present in mammalian cell. Antimicrobial agents may have a low or high selective toxicity (Boyd, 1995). In spite of the great attention to microorganisms living under extreme environmental conditions, including thermophiles and numerous studies of their physiology, genetics, and biochemistry (Ghauri *et al.*, 1991). The secondary metabolism of thermophilic microorganisms is poorly understood. Antimicrobial secondary metabolites occur in some *species* of thermophilic actinomycetes, but virtually nothing is known of thermophilic *Bacilli* producing antibiotic substances, including peptides (Esikova *et al*., 2002). Most of the peptide antibiotics produced by *Bacilli* are active against gram-positive bacteria; however,

**Corresponding author*: Tel: +92-51- 2601022, Fax: +92-51-90643157, e-mail: safiamrl@yahoo.com

compounds such as Polymyxin, Colistin, and Circulin exhibit activity almost exclusively upon gram-negative bacteria, where as Bacillomycin, Mycobacillin, and Fungistatin are effective against molds and yeasts (Katz and Demain, 1997). Keeping in mind the importance of antibiotics in the current era, the ambitious objectives of the present research work was to check the ability of thermophilic isolates for the production of antibacterial compound and further to optimize the fermentation conditions for the production of these antimicrobials by the selected bacterial *specie*.

MATERIALS AND METHODS

Screening of antibiotic producing Bacillus species

Thermophilic bacterial strains were initially isolated from different areas of Sindh Province, and these samples were used and checked for antibacterial activity against *Micrococcus luteus* and *Staphylococcus aureus.* Plates were incubated at 37°C for 24 hours.

Identification of *Bacillus species*

Isolated colony was identified on the basis of routine morphological and biochemical tests (Buchanan and Gibbons, 1974).

Biochemical characteristics

The isolate was biochemically characterized according to the Bergey's Manual of Determinative Bacteriology and these tests including indole production test, citrate utilization test, oxidase test, catalase test, starch hydrolysis, casein hydrolysis, and carbohydrate fermentation test were performed to check the biochemical characteristics of producing strain (Bergey and Holt, 1994).

Antibiotic production by Bacillus species

The thermophilic *Bacillus specie* was checked for antibiotic activity, initially *Bacilli* strain was taken and their antimicrobial activity was evaluated. *Bacilli specie* strain SAT4 was screened and selected. The following steps were taken in this regard.

Inoculum preparation

Inoculum was prepared in nutrient broth. 100ml of media was prepared in 250ml flask and autoclaved at 121°C and 15psi pressure for 15minutes, antibiotic producing thermophilic *Bacillus specie* was grown on nutrient agar plate and incubated at 50°C for 24 hours. Next day, the flask was inoculated with a fresh culture of *Bacillus specie* by using the sterilized loop and incubated again at 50°C for 24 hours in an orbital shaker at 150rpm.

Batch fermentation

Synthetic media having, 5.0g of L-glutamic acid, 0.5g of KH_2PO_4, 0.5g of K_2HPO_4, 0.2g of MgSO4.7H2O, 0.01g of $MnSO_4$.H2O, 0.01g of NaCl, 0.01g of $FeSO_4.7H_2O$, 0.01g of $CuSO_4.7H_2O$, 0.015g of $CaCl_2.2H_2O$ in 1 liter distilled water, was used as production medium (Bushra *et al.*, 2007). Sterilized medium (100ml) was added in 250ml flask, pH was adjusted to 7. Glucose solution (1%) was sterilized through 0.2µm filter paper and added to flask. Inoculum (10%) was taken in the respective flask. Flask was incubated again at 55°C in orbital shaker at 150rpm. After every 24 hours, samples were taken in eppendorff tubes up to 144 hours, centrifuged for 10 minutes at 10,000rpm to get cell free supernatant. The pellets were discarded and the supernatant was filtered through 0.2µm filter paper and stored in refrigerator.

Agar well diffusion assay

Agar well diffusion assay was used to check the production of antimicrobial metabolites (Sen *et al*., 1995). Test cultures of *Micrococcus luteus* and *Staphylococcus aureus* were grown to 0.5 McFarland standard of optical density and spread on agar plates in the form of lawn. With the help of sterilized borer wells were made in the agar plates and culture filtrate of the producing organism was poured in the well and incubated for 24 hours, zone of inhibition was measured for the activity of antimicrobial compounds.

Optimization of various parameters for maximum antibiotic production

Effect of incubation period

The strain was incubated at 50°C in an orbital shaker at 150rpm and samples were taken 24 hourly from 0 to 144 hours. The cell free supernatant of sample taken at different times was used against *Staphylococcus aureus* and *Micrococcus luteus* and activity was measured in terms of zone of inhibition.

Effect of pH

Effect of pH for optimum antibiotic production was studied by inoculating the organism in the synthetic media adjusted to different pH values (4, 5, 6, 7, 8 and 9). Samples were drawn 24 hourly from 0 to 72 hours, centrifuged and supernatants were used for analysis of antimicrobial activity.

Effect of nitrogen concentrations

The effect of different concentration of L-glutamic acid as nitrogen source (0.25, 0.5, 1, 1.5 and 2%) in production media was studied by inoculating the organism in these media. Samples were drawn 24 hourly from 0 to 72 hours, centrifuged and supernatants were used for analysis of antimicrobial activity.

Effect of different glucose concentrations

Glucose concentration was varied from 0.25 to 3% in the production media to optimize the maximum antibiotic production by *Bacillus specie.*

Effect of temperatures

Effect of temperature on growth of thermophilic strain in synthetic media was checked for the optimum production of antimicrobials by incubating the production media, inoculated with bacterial strain, at various temperatures (45, 50, 55, and 60°C). Samples collected at various time intervals were processed for antibacterial assay (Awais *et al*., 2008).

RESULT

Identification of thermophilic specie

The thermophilic strain was taken from Microbiology Research Lab, which was originally taken from certain parts of Sindh. The strain was identified on the basis of standard, morphological and biochemical methods, according to Bergey's Manual of Determinative Bacteriology, and found to be *Bacillus specie*. The bacterial isolate was gram positive rods, spore forming and has motility. Biochemical tests indicated that citrate utilization, oxidase, starch hydrolysis and casein hydrolysis were positive while indole, catalase and gas production tests were negative with the positive fermentation of glucose showing acid production (table 1). These tests identified the strain as *Bacillus specie* strain SAT4.

Table 1: Morphological and Biochemical identification tests for bacterial isolate SAT4

Test	Result
Grams Staining	+
Shape	Rods
Spore formation	+
Motility	+
Indole Production	–
Citrate Utilization	+
Oxidase Test	+
Catalase Test	–
Starch Hydrolysis	+
Casein Hydrolysis	+
Gas Production from Glucose	Gas production –ive, Acid production +ive

Antibiotic production by Bacillus specie

Samples drawn during batch fermentations were subjected to agar diffusion assay, using *Micrococcus luteus* and *Staphylococcus aureus* as test organisms. Antimicrobial activity was measured in terms of zone of inhibition. The incubated samples of different time (0 hour to 144 hour) interval were evaluated and optimum antimicrobial activity of inoculum of thermophilic *Bacilli specie* SAT4 was ensured at 48hours (table 2). It was observed that *Bacillus* metabolites showed activity against *Staphylococcus aureus* measured as zone of inhibition of 24mm while *Micrococcus luteus* showed as 25mm zone. It was observed that after 48hour, there was gradual decrease of activity and less zones were obtained at 144hours against both indicator strains.

Optimization of various parameters for maximum antibiotic production

Parameters like time of incubation, pH of the medium, glucose concentrations, nitrogen concentrations and temperature were optimized for the production of antibiotic by thermophilic *Bacillus specie* strain SAT4.

Effect of time of incubation

The *Bacillus* SAT was taken and introduced into the production media. The production was carried out at 55°C in an orbital shaker at 150rpm and samples were withdrawn 24 hourly from 0 to 72 hours. Antimicrobial activity was measured in terms of zone of inhibition. Best activity (24mm) was shown after 48 hours, while after 24 hours 22mm of zone of inhibition was recorded and then there was gradual decrease with passage of time against *Micrococcus luteus* by using the synthetic media while best activity (18mm) was observed at 24 and 48 hours with gradual decrease after passage of time against *Staphylococcus aureus* (table 2).

Table 2: Antimicrobial activity in fermentation broth of thermophilic *Bacillus specie* SAT4 at different time interval, against *Micrococcus luteus* and *Staphylococcus aureus*

Time (hours)	Zone of inhibition produced by thermophilic *Bacillus specie* SAT4 (mm)	
	Staphylococcus aureus	*Micrococcus luteus*
0	–	–
24	22	25
48	24	25
72	18	22
96	15	16
120	10	14
144	7	10

Effect of pH

Effect of pH on the production of antibiotics was studied by inoculating the test strain in the medium at different pH. Best activity was observed at pH 5 by thermophilic strain against both indicator strains, while second best activity was observed at pH 4 and there was gradual decrease of activity with the increase of pH and minimum activity was seen at pH 9 against both indicator strains (fig. 1).

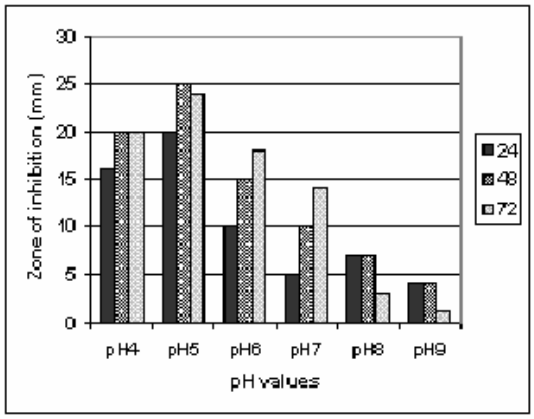

Fig. 1: Antimicrobial activity against *Micrococcus luteus* at different pH values

Effect of different glucose concentrations

Glucose concentration was varied from 0.25 to 3% in the production medium for thermophilic strain and checked for maximum antibiotic production. The best anti-microbial activity was observed by using the 2% glucose concentration at 48 hours. The 26mm zone of inhibition was formed against *Micrococcus luteus* while 22mm zone of inhibition was observed against *Staphylococcus aureus* (fig. 2). Comparatively *Staphylococcus aureus* showed less sensitivity at these concentrations.

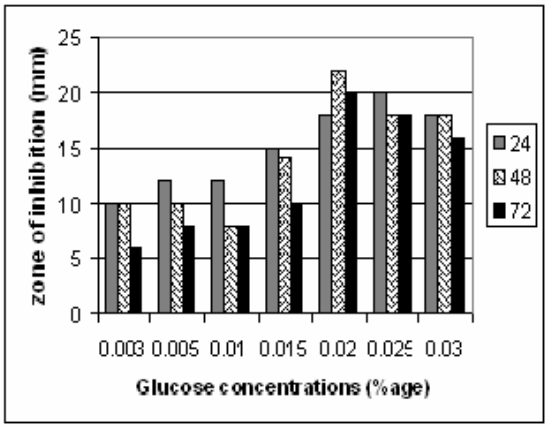

Fig. 2: Antimicrobial activity against *Staphylococcus aureus* at different glucose concentrations

Effect of different nitrogen concentrations

Glutamic acid was used to evaluate the nitrogen concentration which was varied from 0.25 to 2% in the production medium. Best activity was found by using the 1.5% of glutamic acid and maximum zones were observed (26mm) at 48 hours against *Micrococcus luteus* (fig. 3) while zones of 22mm activity was shown against *Staphylococcus aureus* at the same conditions. The second best activity was found when 2% glutamic acid was used. The activity was decreased with the passage of time.

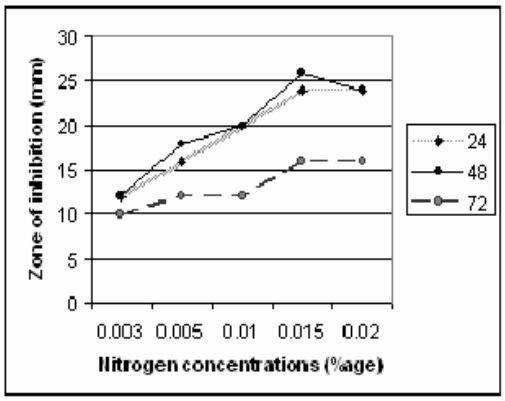

Fig. 3: Antimicrobial activity against *Micrococcus luteus* at different nitrogen concentrations

Effect of different temperatures on antibiotic production

Effect of different temperatures (45, 50, 55, and 60°C) on the production of antimicrobial in fermentation broth was studied. Best activity was shown at temperature 55°C by thermophilic *specie* against both indicator strains, while second best activity was observed at 50°C (fig. 4 and table 4).

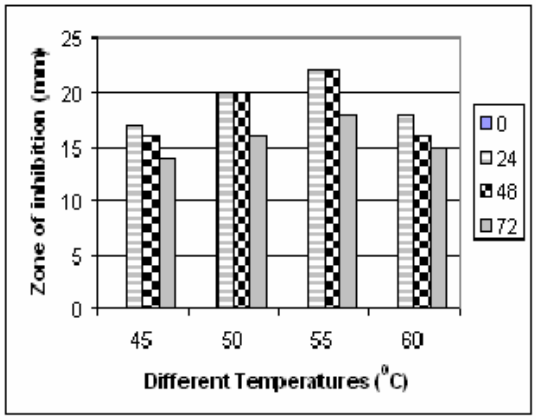

Fig. 4: Antimicrobial activity against *Staphylococcus aureus* at different temperatures

DISCUSSION

The antibiotics are getting worth now a day and due to number of clinical concerns their usage is important and there is still need of research for the new discoveries. There are many strains of genus *Bacillus* which can produce a wide variety of antibiotics including polypeptide antibiotics Bacitracin, Polymyxin, Subtilin, etc. Several bacitracins have been characterized; Bacitracin A is the dominant commercial product (Schallmey *et al*., 2004).

The thermophilic bacterial isolates from the Thar Desserts, Sindh Province showing antimicrobial activity against other organisms were screened and one selected

Table 4: Antimicrobial activity in broth of *Bacillus* specie SAT4 at different temperatures against *Micrococcus luteus* and *Staphylococcus aureus.*

Time	Zone of Inhibition (mm) against *Micrococcus luteus* at Temperature				Zone of Inhibition (mm) against *Staphylococcus aureus* at Temperature			
	45°C	50°C	55°C	60°C	45°C	50°C	55°C	60°C
0	–	–	–	–	–	–	–	–
24	18	22	25	19	17	20	22	18
48	18	21	24	17	16	20	22	16
72	16	18	20	16	14	16	18	15

bacteria was identified. Morphological and biochemical characteristics showed that bacteria belong to *Bacillus specie.* That thermophilic strain was identified according to the Bergey's Manual of Determinative Bacteriology (Bergey and Holt, 1994) and identified as *Bacillus specie* SAT4. Bacteria belonging to the genus *Bacillus* have a long and distinguished history in the realms of biotechnology. *Bacilli* are also sources of numerous antibiotics, flavor enhancers such as purine nucleosides, surfactants, and various other products (Priest, 1992).

The traditional approach to the identification of *Bacilli* was based on the morphological groups mentioned. The bacterium was identified to the genus and species level using a panel of physiological and biochemical tests. Gram-positive, rod-shaped bacteria that differentiate into heat-resistant endospores under aerobic conditions are placed in the genus *Bacillus* (Priest, 1992).

In this research study, the antibacterial spectrum of the *Bacillus* strain was tested against variety of organisms but they showed best activity against gram positive bacteria like *Staphylococcus aureus* and *Micrococcus luteus* through well diffusion assay. There was no activity against *Pseudomonas auroginosa*. *Bacillus subtilis* strains produce a broad spectrum of bioactive peptides with great potential for biotechnological and biopharmaceutical applications. A well-known class of such compounds includes the Lipopeptides Surfactin, Fengycin, compounds (Vanittanakom *et al*., 1986), iturin A, B and C (Besson *et al*., 1978), mycosubtilins (Peypoux *et al*., 1978), and bacillomycins (Peypoux *et al*., 1984), which are amphiphilic membrane-active biosurfactants and peptide antibiotics with potent antimicrobial activities. Mendo isolated a strain of *Bacillus subtilis* from sugar cane fermentation, which produced a polypeptide antibiotic, bacitracin, which inhibited the growth of *Micrococcus flavus* (Mendo *et al.*, 2004).

When in shake flask liquid fermentation for antimicrobial production by the selected bacterial isolates *Bacillus specie* SAT4 was studied, maximum production was after 48 hours of incubation, which further reduce till 144 hours at 55°C. Best activity was shown after 24 h by *B. pumilus* against *S. aureus* and *M. luteus*, while at 48 h an increased activity was observed only against *M. luteus* (18 mm) (Bushra *et al.,* 2007). Awais *et al*., 2008 also described the antibacterial production by *Bacillus pumilus* was after 48 hours of incubation. Muazz *et al*, 2007 also showed that concentration and inhibitory compound was high at 24 hours in post exponential phase and reached at maximum at 48 hours, in stationary phase by *Bacillus subtilis* MZ-7.

When effects of pH on production of antimicrobial agents by *Bacillus specie* SAT4 was studied, it was evident from the results that SAT4 showed highest concentration and antimicrobial activity after 48 hours of incubation at pH 5, when tested against *Micrococcus luteus* and *Staphylococcus aureus*. The pH of media between 7-8 had antimicrobial production, produced by *Bacillus subtilis* MZ-7 (Muazz *et al.,* 2007). Best activities against *Micrococcus luteus* were shown at pH 8, while at pH 6 and 7, starting from maximum activity, a decrease in activities was observed until 96 h, which again increased at 120 and 144 h. At pH 9, second best activities were observed (Awais *et al*., 2008).

The effect of glucose concentration on the production of antibiotic was studied and it was shown that increased concentration have positive effects on the production of antibiotics. Maximum zone of inhibition was produced against *Micrococcus luteus* at 48 hours by using the 2% glucose. Glucose which is usually an excellent carbon source for bacterial growth interferes with the synthesis of many secondary metabolites. In some microorganisms, the inhibitory effect of glucose has been related to a decrease in pH and this reduction is due to acidification as a result of the accumulation of organic acids (Espeso *et al*., 1993). Activity increased gradually as the glucose concentration was increased, and at 5% glucose the maximum zone of inhibition was observed. *Bacillus pumilus* showed best activity at 48 h at all of the glucose concentrations and a continuous increase in activity from 1 to 5% glucose at 96 h against *S. aureus* (Awais *et al*., 2008).

The nitrogen source (glutamic acid) concentration effected the production of antibiotic and it was shown that increased concentration improve the production of antibiotics. Maximum zone of inhibition was produced against *Micrococcus luteus* and *Staphylococcus aureus* at 48 hours by using the 1.5% glutamic acid for the estimation of nitrogen. It was observed that zones of activity were reduced with gradual decrease in concentration of nitrogen source. Addition of ammonium succinate to the fermentation medium markedly increased the antibiotic productivity as the growth rate was low. Depending on the biosynthetic pathways involved, nitrogen sources may affect antibiotic formation (Gesheva *et al.,* 2005).

Effect of different temperatures (45, 50, 55, and 60°C) on the production of antibiotics was studied by inoculating the test strain in the medium. Antimicrobial activity was measured in terms of zone of inhibition. Best activity was shown at temperature 55°C by thermophilic strain against both indicator strains, while second best activity was shown at 50°C. *Bacillus species* continue to be dominant bacterial workhorses in microbial fermentations. *Bacillus species* are poised to become the preferred hosts for the production of many new and improved products as we move through the genomic and proteomic era (Schallmey *et al*., 2004).

CONCLUSION

The results suggest that thermophilic *Bacillus specie* SAT4 may be a good producer of antibiotics, stable at higher temperature and having antimicrobial activity against Gram-positive bacteria. Moreover maximum activity was observed for *Staphylococcus aureus* and *Micrococcus luteus* at 48 hour of incubation at 55°C at an acidic pH of 5.0 with 2% glucose.

REFERENCES

Awais M, Pervez A, Qayyum S and Saleem M (2008). Effects of glucose, incubation period and pH on the production of peptide antibiotics by *Bacillus pumilus. Afri. J. Microbiol.Res.,* **2**: 114-119.

Berdy J (1980). Recent advances in and prospects of antibiotic research. *Process Biochem*. **15**: 28-36.

Bergey DH, Holt JG (1994). *In*: Bergey's Manual of Determinative Bacteriology. 9th ed. Williams & Wilkins Publishers, Baltimore.

Besson F, Peypoux F, Michel G and Delcambe L (1978). Identification of antibiotics of iturin group in various strains of *Bacillus subtilis. Antibiotic* (Tokyo) **31**: 284-288.

Boyd RF (1995). Chemotherapy, 8th Chapter, Basic Medical Microbiology, Little Brown and Company, Inc. pp.104-125.

Bushra J, Hasan F, Hameed A, Ahmed S (2007). Isolation of *Bacillus subtilis* MH-4 from soil and its potential of polypeptidic antibiotic production. *Pak. J. Pharm. Sci.*, **20**(1): 26-31.

Demain AL, Aharonowitz Y, Martin JF (1983). Metabolite control of secondary biosynthetic pathways. *In*: Vining LC (Ed.), Biochemistry and Genetic Regulation of Commercially Important Antibiotics. Addison-Wesley, London, pp.49-67.

Espeso EA., Tilburn J, Arst HN and Peñalva MA (1993). pH regulation is a major determinant in expression of a fungal penicillin biosynthetic gene. *EMBO J.,* **12**: 3947-3956.

Esikova TZ, Temirov YV, Sokolov SL and Alakhov YB (2002). Secondary Antimicrobial Metabolites Produced by Thermophilic *Bacillus* spp. Strains VK2 and VK21. *Appl. Biochem. Microbiol.,* **38**: 226-231.

Gesheva V, Ivanova V and Gesheva R (2005). Effects of nutrients on the production of AK-111-81 macrolide antibiotic by *Streptomyces hygroscopicus. Microbiol. Reds.,* **160**: 243-248.

Ghauri MA and Johnson DB (1991). Physiological diversity amongst some moderately thermophilic iron-oxidising bacteria**.** *FEMS Microbiol. Ecol.,* **85**: 327-331.

Katz E and Demain AL (1997). The peptide antibiotics of *Bacillus*, chemistry, biogenesis, and possible functions. *Bacteriol Rev*., **41**: 449-474.

Mendo S, Faustino NA, Sarmento AC, Amado F and Moir AJ (2004). Purification and Characterization of a new peptide antibiotic produced b a thermo tolerant *Bacillus licheniformis* strain. *Biotechnol Lett.,* **26**(2): 115-125.

Muaaz MA, Sheikh MA, Ahmad Z and Hasnain S (2007). Production of surfactin from *Bacillus subtilis* MZ-7 grown on pharmamedia commercial medium. *Microbial Cell Fact.,* **10**: 6-17.

Peypoux F, Guinand M, Michel G, Delcambe L, Das BC and Lederer E (1978). Structure of iturin A, a peptidolipid antibiotic from *Bacillus subtilis. Biochemistry*, **17**: 3992-3996.

Peypoux F, Pommier MT, Das BC, Besson F, Delcambe L and Michel G (1984). Structures of bacillomycin D and bacillomycin L peptidolipid antibiotics from *Bacillus subtilis*. *J. Antibiot*., **77**: 1600-1604.

Priest FG (1992). Biology of *Bacilli. In*: *Bacilli* applications to industry. Edited by Doi RH and McGloughlin M. Butterworth-Heinemann, Boston, Mass, pp.293-320.

Schallmey M, Singh A and Ward OP (2004). Developments in the use of *Bacillus species* for industrial production. *Can. J. Microbial.,* **50**: 1-17.

Sen KS, Haque FS and Pal CS (1995). Nutrient Optimization for production of broad spectrum antibiotics by *Streptomyces.* Antibiotics Str. 15.4. *Acta. Microbial. Hung*., **42**: 155-162.

Tortora GJ, Funke BR and Case CL (1995). Antimicrobial Drugs, Microbiology an Introduction, 20th Chapter. The Benjamin/Cummings Publishing Company, Inc., pp.491-514.

Vanittanakom N, Loeffler W, Koch U and Jung G (1986). Fengycin-a novel antifungal lipopeptide antibiotic produced by *Bacillus subtilis* F-29-3. *J. Antibiot.* (Tokyo), **39**: 888-901.

Zinsser H (1988). Antimicrobial agents, 9th chapter. *In:* Zinsser H, Joklik WK, Willett HP, Amos DB, Wilfert C (ed.), Zinsser Microbiology, Prentice Hall International, UK, pp.128-160.