ORIGINAL ARTICLE

ISOLATION OF *BACILLUS SUBTILIS* MH-4 FROM SOIL AND ITS POTENTIAL OF POLYPEPTIDIC ANTIBIOTIC PRODUCTION

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ABSTRACT

The genus *Bacillus* produces mainly polypeptide antibiotics such as bacitracin and polymyxin. *Bacillus* species were isolated from soil by soil sprinkle technique. And all were screened for the production of antibiotic. *Bacillus subtilis* MH-4 gave the maximum antimicrobial activity so finally selected for optimization. During optimization of culture conditions for *Bacillus subtilis* MH-4 best antibacterial activity was obtained at 96 hours of incubation period, at pH-8 and by using glycerol as carbon and L-glutamic acid as nitrogen source. Optimium temperature for antibiotic production was 37^{o}C. The antibiotic was confirmed to be bacitracin by paper chromatography. Antibiotic was further extracted successfully with 1-Butanol, and aqueous concentrate showed activity of 0.8mg/ml. The antibiotic so produced was found to be narrow spectrum active against only Gram-positive bacteria.

Keywords: bacitracin, *Bacillus subtilis*, paper chromatography.

INTRODUCTION

Antibiotics are low molecular-weight (non-protein) molecules produced as secondary metabolites, mainly by microorganisms that live in the soil. A natural assumption is that soil microbes produce antibiotics in their natural habitat and use them to gain advantage over their competitors; that is, antibiotics are presumed to be involved in naturally occurring amensal relationship in the soil. Regardless of the toxicity of some antibiotics produced by bacteria from Bacillus genus to the cells of mammals (e.g. polymyxines, bacitracin, etc.), they were and continued to be in the focus of attention of scientists. The amount of antibiotics produced by these bacilli was approaching 167. From that more, than 66 derived from *B. subtilis* and about 23 originated from *B. brevis* (Katz and Demain, 1977). As is generally recognized, these antibiotics are mainly polypeptides. Most of the peptide antibiotics produced by *Bacillus* are active against gram-positive bacteria (Ming and Epperson, 2002). However, compounds such as polymyxin, colistin, and circulin exhibit activity almost exclusively upon gram-negative forms, whereas bacillomycin, mycobacillin, and fungistatin are effective agents against molds and yeasts (Katz and Demain, 1977). Peptide antibiotics fall into two broad classes whose evolutionary biology is very different. The first is a large and heterogeneous category of peptides that are synthesized on very large, modular enzyme complexes (Peptide synthetases) by bacteria and fungi (Stachelhaus *et al.,* 1996; Konz and Marahiel, 1999; Eppelman *et al.,* 2001). Since these and other synthetases complexes possess enzymatic activities toward the syntheses of secondary metabolite, thus are potential target for drug discovery in the production of potential bio-active peptide and polyketide as well as their hybrids (Mootz and Maraheil, 1999; Maraheil, 1997). They often incorporate unusual amino acids, and may be modified by glycosylation or ring formation or in other ways. This group includes the bacitracin, gramicidin, polymyxin, streptogramin and their derivatives. The second category is quite different. It comprises linear peptides consisting almost entirely of conventional amino acid residues that are produced by all major kinds of organisms (including microbes). These are translated using ribosomes in the usual fashion of protein synthesis, and we therefore call them RAMPs, for Ribosomally synthesized Anti Microbial Peptides (Hancock and Chapple, 1999) to distinguish them from the non-RAMPs of the first category. RAMPs include the defensins, indolicidins and cathelicidins in mammals; bombinin, magainin and buforin in frogs; cecropin and melittin in insects; the thionins in plants; and the bacteriocins, epidermidins and nisin in bacteria. The non-RAMPs are used like conventional antibiotics, and share their strengths and weaknesses while having some of their own (Bell and Gouyon, 2003). Many peptide antibiotics have novel structural motifs, such as cyclic structures, contain uncommon amino acids, especially D-form amino acids, and are often further modified (such as in β-lactam antibiotics) and conjugated with sugars, lipids, and other molecules (Katz, 1968; Katz, 1971; Katz and Demain, 1977). Depending on their amino acid components and

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their conjugates, the mechanisms of their actions may vary dramatically (McCafferty *et al.,* 1999). Examples of peptide antibiotics include some well known or commonly used drugs, such as bacitracin, polymyxin, amphomycin, actinomycin, gramicidin, vancomycin, penicillin, and cephalosporin (D'Aversa *et al.,* 1997). Peptide antibiotics form a unique group of "bio-active molecules" (Hancock and Chapple, 1999). The present study was designed to check the ability of *Bacillus* spp isolates for the production of antibiotics and to optimize different physical and chemical parameters for antibiotic production and to identify, extract and concentrate the so produced antibiotic.

MATERIAL AND METHODS

In the present study soil sprinkle technique was used to isolate antibiotic producing microorganisms. For this purpose about 20-30 particles of soil (less is better than more) from different locations were sprinkled on the surface of nutrient agar plates seeded with the test organism. The plates were incubated at 37°C for 24 hours. Antibiotic activity was checked by zone of inhibition, surrounding a colony. Apparently different colonies having zones of inhibition were picked and streaked on separate nutrient agar plates to get pure cultures and morphological identification was done by Gram-staining. These isolates were used as the source of antibiotic producing microbes. All strains were stored at 4°C and refreshed periodically for the study purpose Synthetic media having, 5.0g of L-glutamic acid, 0.5g of KH_2PO_4, 0.5g of K_2HPO_4, 0.2g of $MgSO_4.7H_2O$, 0.01g of $MnSO_4.H_2O$, 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 litre distilled water (pH 7.0), was used as production medium. After sterilization of synthetic media concentrated glucose solution previously sterilized by syringe filtration was added to give a final concentration of 1% in the medium. 72 hours old inoculum prepared in nutrient broth was used at concentration of 10%. Shake flask fermentation method (37°C at 150 rpm for 96 hours) was used for antibiotic production. After 96 hour cultivation media was centrifuged at 10000rpm for 20 minutes at 4°C to get cell free supernatant. The pellet was discarded and sterilized supernatant was used for agar well diffusion assay. Antibiotic so produced was identified via paper chromatography. Ascending chromatogram on Whatman No. 1 paper were developed in glass jars containing 1 cm of solvent mixture. Samples were spotted 2 cm above the base of the paper, with a hypodermic syringe and dried thoroughly before placing in the solvent. After the solvent had migrated to the top of the paper (12 cm) the chromatogram were dried thoroughly. Exposure to steam intermittently during the drying ensured adequate removal of acetic acid. The migrated antibiotics were detected bioautographically. Papers were placed for one-half hour on nutrient agar plates seeded with the test organism. Plates were incubated at 37°C for 17 hours. Solvent composition and concentration used was as follows. Acetone, Acetic acid and H_2O at 20:06:74 ratios. The clarified harvest was extracted twice, using one half volume of 1-butanol for each of two extractions. The 1-butanol was then decanted, filtered, and distilled in vacuum in the presence of water until all butanol was removed and an aqueous concentrate was obtained. It was then assayed for antibiotic concentration by using agar well diffusion method.

RESULTS

By using soil sprinkle technique 12 apparently different colonies having zones of inhibition were picked and streaked on separate nutrient agar plates to get pure cultures and cell morphologically was studied by Gram-staining. 11 of them were Gram- positive Bacilli, having big colonies, few strains had slight pigmentation, few strains had branching morphology and mostly had uneven margins. Growth was very abundant and rapid.

Out of all the tested strains MH-4 was found to be most suitable strain so it was selected for optimization. It was identified to be *Bacillus subtilis*. The identification was made according to the key of Bergey's manual of determinative bacteriology (Holt *et al.,* 1994). During optimization of various parameters it was observed that the maximum antibiotic production was obtained after 96 hours of incubation. Although significantly good amount was also found at 3-5 days of incubation period however after that it started declining (fig. 1).

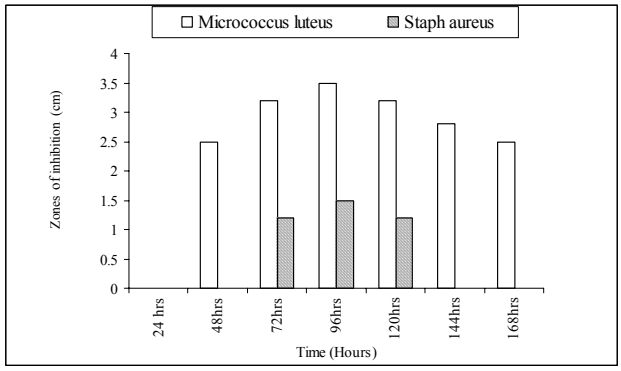

Fig 1: Optimization of incubation time for the production of antibiotic from *B. subtilis* MH-4.

Maximum antibiotic production was obtained at alkaline condition of pH 8 and significant amount of antibiotic was also produced at pH 6-9 (fig. 2). At pH 5 a small zone of inhibition only against *Staph aureus* was achieved. However pH 8 was used for further optimization experiments.

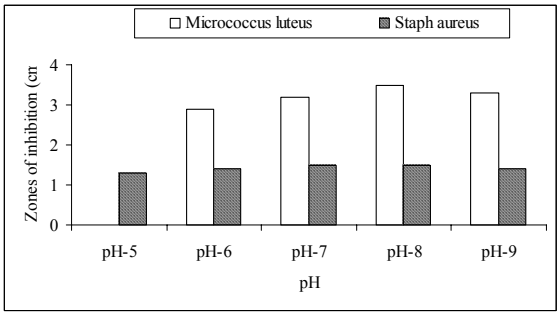

Fig 2: Optimization of pH for the production of antibiotic from *B. subtilis* MH-4.

Maximum antibiotic production was obtained when glycerol was used as carbon source. However significant antibacterial activity was also achieved when maltose, sorbitol, lactose, glucose, and acetic acid were employed as carbon sources (fig. 3). For further experiments glycerol was used as carbon source.

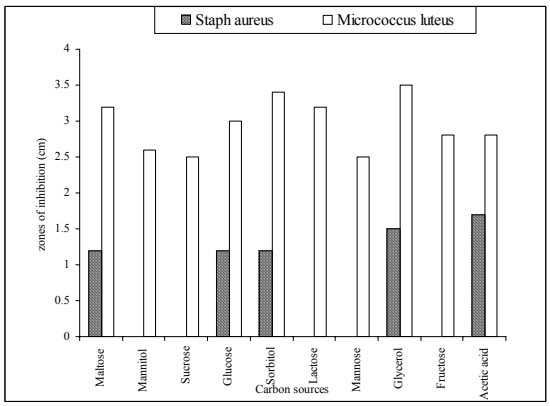

Fig 3: Optimization of carbon sources for the production of antibiotic from *B. subtilis* MH-4

The antibiotic production was maximum when L-glutamic acid was used as nitrogen source (fig. 4). Next best source was found to be alanine, and no activity was observed when $NaNO_3$ and NH_4Cl were used as nitrogen sources. For further experiments L-glutamic acid was used as nitrogen source.

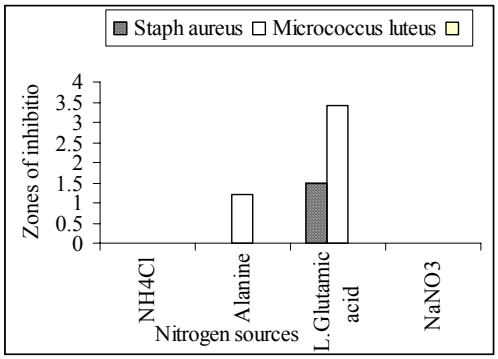

Fig 4: Optimization of nitrogen sources for the production of antibiotic from *B. subtilis* MH-4.

The antibiotic production at different temperatures was depending on test organism (fig. 5). Against *Staph aureus* best activity was achieved at 30°C and against *Micrococcus luteus* best activity was achieved at 37°C. Against *Micrococcus luteus* considerably good activity was achieved even at 50°C. However for further experiments 37°C temperature was used.

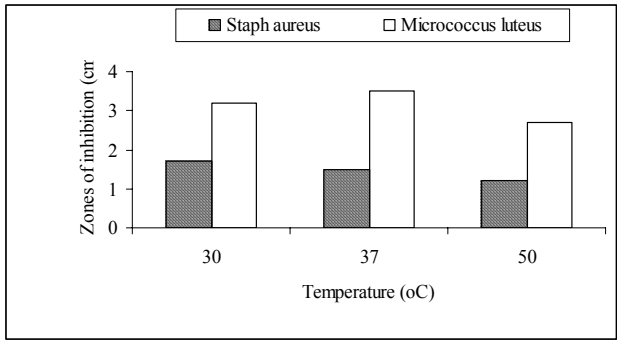

Fig 5: Optimization of temperature for the production of antibiotic from *B. subtilis* MH-4.

Rf value was calculated for migrated antibiotics it was calculated to be 0.97 and was found to be for bacitracin (Snell *et al.,* 1955).

Zones of inhibition achieved by aqueous concentrate were against *Staph aureus* 3.2mm and against *Micrococcus luteus* it was 4.1mm. After extraction and purification 0.8mg/ml of bacitracin was received, calculations were made according to standard curve.

DISCUSSIONS

The present study was carried out to study the production of antibiotic from newly isolated *Bacillus* species. Different bacteria were isolated from soil and identified as *Bacillus.* All showed antibacterial activity against various pathogens. Antibiotic production is a feature of several kinds of soil bacteria and fungi and may represent a survival mechanism whereby organisms can eliminate competition and colonize a niche (Talaro and Talaro, 1996; Jensen and Wright, 1997). Members of the genera *Bacillus*, *Streptomyces,* and *Pseudomonas* are soil bacteria that produce a high proportion of agriculturally and medically important antibiotic (Yoshiko *et al.,* 1998; Sharga *et al.,* 2004). Rhizobacteria are present in the soil in an average of about 10^8 cells per gram (Stein, 2005). The potential of *B. subtilis* to produce antibiotics has been recognized for 50 years. Peptide antibiotics represent the predominant class. However, systematic studies that survey the complete spectrum of antibiotic activities by different *B. subtilis* strains are rare (Pinchuk *et al.,* 2002).

Altogether, it seems to be that *B. subtilis* is outstanding in the genus *Bacillus* with regards to its potential to produce

so many different antibiotics. However, *B. subtilis* is by far the most commonly investigated *Bacillus* genus, and the large number of known *B. subtilis* antibiotics might reflect the numerousness of natural isolates and studies. Also other *Bacilli* such as *Bacillus brevis* (brevistin, edeines, gramicidines, tyrocidin) or *B. amyloliquefaciens* (Koumoutsi *et al.,* 2004) produce a couple of antibiotics, although their number seems to minor as compared with *B. subtilis*.

In the present study synthetic media containing all essential minerals, carbon and nitrogen sources was used as production media. When nutrient broth was used as production media, not significant antibiotic was produced because nutrient broth was not fulfilling all requirements necessary for antibiotic production. This shows that certain metal ions are required for the activity of bacitracin. Although most antibiotics do not need metal ions for their biological activities, there are a number of antibiotics that require metal ions to function properly, such as bleomycin, streptonigrin, and bacitracin. The coordinated metal ions in these antibiotics play an important role in maintaining proper structure and/or function of these antibiotics. Removal of the metal ions from these antibiotics can cause changes in structure and/or function of these antibiotics. These antibiotics are called metalloantibiotics (Epperson and Ming, 2000; Ming, 2003). Metal ion may be essential for the antimicrobial activity of bacitracin (Weinberg, 1958). Bacitracin production is inhibited by metal chelator EDTA. The inhibitory effect of EDTA was reversed by the addition of excess Mn^{+2}, Co^{+2}, or Zn^{+2} to the culture (Haavik, 1974).

Different parameters for the optimum production of antibiotics from *Bacillus subtilis* MH-4 were optimized in the present study. Incubation time optimization gave 96 hours as optimum time for antibiotic production. As antibiotics are secondary metabolites so these are formed usually when organism has passed rapid growth phase. The same thing was discussed by (Demain, 1972). Demain concluded that the synthesis of peptide antibiotics is initiated after the organism has passed the rapid growth phase. Although antibiotic formation usually follows logarithmic growth (presumably due to some type of repression of antibiotic synthetases in the growth phase), this is not universally observed. It is clear that antibiotics are sometimes produced during growth and that both genetic and nutritional modifications can shift the time of antibiotic synthesis in relation to the growth phase. (Hanlon and Hodges, 1981), concluded that bacteria and proteases may under certain conditions, arise throughout the phase of growth which is truly exponential rather than the phase of active but non exponential growth.

Changes in external pH affect many cellular processes such as the regulation of the biosynthesis of secondary metabolites. Bacitracin production by *Bacillus subtilis* is pH dependent (Montserrat *et al.,* 2000). In present study pH was also optimized, during optimization of pH it was observed that best activity was achieved at pH range of 6-9 with maximum activity at pH 8 and almost no activity was achieved at pH 5. The same results were achieved by (Snoke, 1960). Where the formation of bacitracin by protoplasts of *B. licheniformis* was maximum at pH 8. (Hanlon and hodges, 1981) suggested that antibiotic production during growth may be achieved by preventing the pH drop normally observed after the metabolism of glucose.

In the present study ten different carbon sources were also employed to optimized best carbon source, it was found that antibiotics formation was maximum when glycerol was used as carbon source. Although, considerably good activity was obtained when maltose, glucose, sorbitol and lactose were used as carbon sources. (Qadeer *et al.,* 1988) studied the production of antibiotic bacitracin in starch-glucose-soybean meal medium by *Bacillus licheniformis*. The effect of the replacement of soybean meal by sunflower or pharmamedia and glucose by mannose, sucrose, lactose or beet molasses was investigated. The production of the antibiotic, however, was found to be maximum in the presence of soybean meal and sucrose or mannose. 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. Bacitracin production by *Bacillus subtilis* is pH dependent and that the inhibitory effect of glucose is due to acidification as a result of the accumulation of organic acids (Montserrat *et al.,* 2000).

Four different nitrogen sources were also employed in the present study to optimized best nitrogen source; it was found that antibiotics formation was maximum when L-glutamic acid was used as nitrogen source. (Egorov *et al.,* 1986) concluded that the substitution of glutamate for trypton results in a dramatic deceleration of the bacterial growth and biomass accumulation, and the process of the antibiotic biosynthesis ceases.

Temperature is also an important regulator of the rate of metabolism and the growth rate of microorganisms. A shift in temperature can alter the utilization rate of one component as compared to another, thus unbalancing the medium with respect to growth (Hunt and Stieber, 1986). In the present study temperature optimization of MH-4 was also carried out and it was found that antibiotic formation was dependent on test organism. When *Staph aureus* was used as test organism best inhibition was achieved at 30^{o}C and when *Micrococcus luteus* was used as test organism best activity was achieved at 37^{o}C. And MH-4 also produced significant quantity of antibiotic against *Micrococcus luteus* at 50^{o}C. (Anker *et al.,* 1947)

also studied the production of antibiotic and he concluded that the antibiotic production was maximum at 37°C. (Egorov *et al.,* 1983) also studied the effect of temperatures ranging from 30°C to 55°C on the synthesis of exoprotease and bacitracin, as well as on sporification in *Bacillus licheniformis*. The synthesis of bacitracin is substantially sensitive to the temperature variation. The maximum synthesis of the antibiotic was observed at 50°C.

Bacitracin was extracted successfully with 1-butanol. (Andrea *et al.,* 2001) also extracted bacitracin with n-butanol. The antibiotic produced in present study was confirmed to be bacitracin as it inhibits only Gram positive bacteria, and also confirmed to be bacitracin by paper chromatography. The same results were achieved by (Snell *et al.,* 1955). He used the method of ascending chromatography on whatman No 1. He used different solvents in different concentrations when he used Acetone, Acetic acid and H_2O at 20:06:74 ratio he get Rf value of 0.9. The same result was obtained in our experiment. So the antibiotic produced was confirmed to be bacitracin. Bacitracin has bactericidal activity against Gram positive organisms only (Ming and Epperson, 2002; Reiko *et al.,* 2003). The bacitracin so produced was also found to be narrow spectrum active against only Gram-positive microorganisms.

The findings of the present study led to conclude that the antibiotic producing bacillus strains could easily be isolated from soil by soil sprinkle technique. The *Bacillus subtilis* MH-4 is a best antibiotic producing strain among the tested organisms. The optimum pH for antibiotic production by *B. subtilis* MH-4 was 8, although good activity was also achieved at pH 6-9. The maximum antibiotic activity by *B. subtilis* MH-4 was achieved at 96 hours of incubation period. The optimum temperature for antibiotic production by *B. subtilis* MH-4 was depending upon test organism. 37°C for *Micrococcus luteus* and 30°C for *Staph aureus*. The best carbon source for antibiotic production by *B. subtilis* MH-4 was found to be glycerol although considerably good activity was also achieved with maltose, sorbitol, lactose, and glucose. The best nitrogen source for antibiotic production by *B. subtilis* MH-4 was found to be L-glutamic acid. The antibiotic so produced by *B. subtilis* MH-4 was found to be active only against gram-positive bacteria. It was concluded to be narrow spectrum antibiotic. The antibiotic so produced by *B. subtilis* MH-4 was identified to be bacitracin by paper chromatography. The bacitracin so produced by *B. subtilis* MH-4 was extracted successfully with 1-butanol. *B. subtilis* is considered a benign organism as it does not possess traits that cause disease. It is not considered pathogenic or toxigenic to humans, animals, or plants. The potential risk associated with the use of this bacterium in fermentation facilities is low. So it can safely be used for fermentation and genetic manipulation.

REFERENCES

Andrea MN, Konz D and Marahiel MA (2001). The two-component regulatory system BacRS is associated with bacitracin 'self-resistance' of *Bacillus licheniformis* ATCC 10716. *Eur. J. Biochem*. **268**: 3180-3189.

Anker HS, Johnson BA, Goldberg J and Meleny FL (1947). Bacitracin: methods of production, concentration, and partial purification, with a summary of the chemical properties of crude bacitracin. *J. Bacteriol.,* **56**: 249-255.

Bell G and Gouyon P (2003). Arming the enemy: the evolution of resistance to self proteins. *Microbiology,* **149**: 1367-1375.

D'Aversa G, Stern GA, Zimmerman T, Kooner K and Sharir M (1997). (Eds). Textbook of Ocular Pharmacology, Philadelphia: Lippincott-Raven Publishers, pp.549-552.

Demain AL (1972). Cellular and environmental factors affecting the synthesis and excretion of metabolites. *J. Appl. Chem. Biotechnol*., **22**: 345-362.

Egorov NS, Vybornykh SN, Loriia ZK and Parygin EV (1983). Effect of temperature on exoprotease and bacitracin synthesis and sporulation in *Bacillus licheniformis* 28KA. *Prikl Biokhim Mikrobiol.,* **19**(6): 733-7.

Egorov NS, Loriia ZK, Vybornykh SN and Khamrun R (1986). Effect of the culture medium composition on bacitracin synthesis and sporulation in *Bacillus lichenformis* 28 KA. *Prikl. Biokhim. Mikrobiol.,* **22**(1): 107-11.

Eppelmann K, Doekel S and Marahiel MA (2001). Engineered biosynthesis of the peptide antibiotic bacitracin in the surrogate host *Bacillus subtilis*. *J. Biol. Chem.,* **276**: 34824-34831.

Epperson JD and Ming LJ (2000). Proton NMR Studies of Co (II) Complexes of Bacitracin Analogs – Insight into Structure-Activity Relationship. *Biochemistry,* **39**: 4037-4045.

Haavik HI (1974). Studies on the formation of bacitracin by *Bacillus licheniformis*: Role of catabolite repression and organic acids. *J. Gen. Microbiol*., **84**: 321-326.

Hancock REW and Chapple DS (1999). Peptide antibiotics. *Antimicrob. Agents and Chemother.*, **43**: 1317-1323.

Hanlon GW and Hodges NA (1981). Bacitracin and pretease production in relation to sporulation during exponential growth of Bacillus lichiniformis on poorly utilized carbon and nitrogen sources. *Journal of Bacteriology,* **147**(2): 427-431.

Holt JG, Krieg NR, Sneath PH, Stanley JT and Williams ST (1994). Bergey's Manual of Determinative Bacteriology, 9th Edn., Williams and Wilkins, Baltimore pp.529-550.

Hunt GR and Stieber RW (1986). Inoculum development. In: Manual of industrial microbiology and biotechnology. Eds. Demain Al and Soloman NA. American society for Micro. Washington. D.C., pp.29-45.

Jensen MJ and Wright DN (1997). Chemotherapeutic Agents. *In:* Microbiology for the Health Sciences: Prentice Hall, New York, pp.132-145.

Katz E (1968). Peptide antibiotics: proteins that never grew up? *Lloydia,* **31**: 364-394.

Katz E (1971). Biosynthesis of polypeptide antibiotics. *Pure. Appl. Chem.,* **28**: 551-570.

Katz E and Demain AL (1977). The peptide antibiotics of *Bacillus*: Chemistry, Biogenesis, and possible functions. *Bacteriological Review,* **41**: 449-474.

Konz D and Marahiel MA (1999). How do peptide synthetases generate structural diversity. *Chem. Biol.,* **6**: 39-48.

Koumoutsi A, Chen XH, Henne A, Liesegang H, Hitzeroth G and Franke P (2004). Structural and functional characterization of gene clusters directing nonribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. *J. Bacteriol.,* **186**: 1084-1096.

Marahiel MA (1997). Protein templates for the biosynthesis of peptide antibiotics. *Chem. Biol*., **4**: 561-567.

McCafferty DG, Cudic P, Yu MK, Behenna DC and Kruger R (1999). Synergy and Duality in Peptide Antibiotic Mechanism. *Curr. Opin. Chem. Biol.,* **3**: 672-680.

Ming LJ and Epperson JD (2002). Metal Binding and Structure-Activity Relationship of the Metalloantibiotic Peptide Bacitracin. *Journal of Inorganic Biochemistry,* **91**: 46-58.

Ming LJ (2003). Structure and function of "metalloantibiotics". *Med. Res. Rev.,* **23**(6): 697-762.

Montserrat S, Rius N and Loren JG (2000). Rapid extracellular acidification induced by glucose metabolism in non-proliferating cells of *Serratia marcescens. Intl. Microbiol*., **3**: 39-43.

Pinchuk IV, Bressollier P, Sorokulova IB, Verneuil B and Urdaci MC (2002). Amicoumacin antibiotic production and genetic diversity of *Bacillus subtilis* strains isolated from different habitats. *Res. Microbiol.,* **153**: 269-276.

Qadeer MA, Younus O, Ashfaq SR and Khan FZ (1988). Production of bacitracin by *Bacillus licheniformis*. *Pak. J. Sci. Ind. Research,* **31**(1): 30-34.

Reiko O, Tateno K, Okada Y, Okajima H, Asai K, Sadaie Y, Murata M and Aiso T (2003). A bacitracin-resistant *Bacillus subtilis* gene encodes a homologue of the membrane- spanning subunit of the *Bacillus licheniformis* ABC transporter. *J. Bacteriol.,* **185**(1): 51-59.

Sharga BM, Nikolaychuk VI and Maga MI (2004). Comparative IR Spectrometry of antimicrobial substances derived antibiotic from Bacillus. MIKPOBIOJIOTIR, **15**: 75-77

Snell N, Ijichi K and Lewis JC (1955). Paper chromatographic identification of polypeptide gram positive inhibiting antibiotics. *Appl. Microbiol.,* **4**: 13-17.

Snoke JE (1960). Formation of bacitracin by protoplast of *Bacillus licheniformis*. *J. Bacteriol.,* **81**: 986-989.

Stachelhaus T, Schneider A and Marahiel MA (1996). Biochemical characterization of peptide carrier protein (PCP). The thiolation domain of multifunctional peptide synthetases. *Chem. Biol.,* **3**: 913-921.

Stein T (2005). Bacillus subtilis antibiotics: structures, syntheses and specific functions. *Molecular Microbiology,* **56**: 845-851.

Talaro A and Talaro K (1996). Drugs, Microbes, Host. The Elements of Chemotherapy In Foundations in Microbiology. W.M. Brown: New York.

Weinberg ED (1958). Enhancement of bacitracin by the metallic ions of group II B. In Antibiotics Annual, 1958/59. Medical Encyclopedia, Inc., New York, 1959. Welch H and Marti-Ibanez F (Ed.), pp.924-929.

Yoshiko H, Okamoto S, Muramatsu H and Ochi K (1998). Acquisition of certain Streptomycin resistant Mutations Enhances antibiotic production in bacteria. *Antimic Agents & Chemotherapy*, **42**(8): 2041-2047.

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