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Production of Antimicrobial Metabolites by *Bacillus subtilis* Immobilized in Polyacrylamide Gel

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Abstract.- Microbial cells can be immobilized on different support matrices to produce a number of metabolites like enzymes and antibiotics. Immobilization is a very useful process because growth and metabolic production can be uncoupled without affecting metabolite yields. The purpose of this study was to optimize fermentation conditions (pH, incubation periods and glucose concentrations) for maximum production of peptide antibiotics from isolated *Bacillus subtilis* immobilized in polyacrylamide gel and screened for the production of antibiotics by shake flask fermentation at 30°C by checking activity against *Micrococcus luteus* (ATCC#10240) through antibiotics diffusion assay. Maximum production of peptide antibiotic was optimized at pH 6-9, incubation time 0-144 hours and glucose concentration 1-5 %. Maximum activity was at pH 8 after 4 hrs of incubation, whereas, activity was different at 0 and 4 hrs, at various glucose concentrations. Activity of antibiotic increased for immobilized cells just after 4 hours of incubation, which shows that immobilization was a better process, as compared to free cell production of antibiotics.

Keywords: *Bacillus subtilis*, immobilization, *Micrococcus luteus*, polyacrylamide gel.

INTRODUCTION

Antibiotics are low molecular-weight (non-protein) molecules produced as secondary metabolites, mainly by microorganisms that live in the soil. Regardless of the toxicity of some antibiotics probably by some *Bacillus* strains to the cells of mammals (*e.g.*, polymyxines, bacitracin, etc.) they continued to be in the focus of attention of scientists.

The amount of antibiotics produced by bacilli was approaching 167, being 66 derived from *B. subtilis*, 23 from *B. brevis* and the remaining peptide antibiotics are produced by other species of genus *Bacillus.* The main antibiotic producers of this genus are *B. brevis* (*e.g.*, gramicidin, tyrothricin), *B. cereus* (*e.g.*, cerexin, zwittermicin), *B. circulans* (*e.g.*, circulin), *B. laterosporus* (*e.g.*, laterosporin), *B. licheniformis* (*e.g.*, bacitracin), *B. polymyxa* (*e.g.*, polymyxin, colistin), *B. pumilus* (*e.g.*, pumulin), *B. subtilis* (*e.g.*, polymyxin, difficidin, subtilin, mycobacillin, bacitracin). As is generally assumed, these antibiotics are mainly polypeptides (Berdy, 1974; D'Aversa and Stern, 1997; Hancock and Chapple, 1999).

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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).

Bacitracin produced by *B. subtilis* is very effective topically and its action is especially on Gram-positive cell walls. The biggest share of industrial enzymes are produced by *Bacillus*, the laundry industry is consuming various subtilisins, cellulases and amylases produced by *B. subtilis* (Jarnagin and Ferrari, 1992). Other uses of enzymes isolated from *B. subtilis* include, modification of milk proteins in dairy products by neutral proteases, starch and maltose syrup production by the different amylases and pullulanases, high fructose corn syrup production utilizing glucose isomerases and modification of the barley cell wall in brewing processes by beta-glucanases (Zukowski, 1992). More over, insecticides, nucleotides and nucleosides (Demain, 1987), and amino acids (Priest, 1989), are produced by various species of *B. subtilis.*

Currently microbial cells are immobilized to produce a number of products like enzymes and antibiotics. Immobilization of microbial cells in biological processes can occur either as a natural

phenomenon or through artificial process. During the last 20–25 years, the cell immobilization technology has attracted the attention of several research groups. Immobilization commonly is accomplished using a high molecular hydrophilic polymeric gel such as alginate, carrageenan, agarose, polyacrylamide, polyester, polystyrene and polyurethane. Many methods namely adsorption, covalent bonding, crosslinking, entrapment, and encapsulation are widely used for immobilization (Ramakrishna and Prakasham, 2007).

Freeman and Aharonowitz (1981) developed a method for the immobilization of whole microbial cells. Cells were suspended in a solution of preformed, linear, water-soluble polyacrylamide chains, partially substituted with acylhydrazide groups.

The present study was designed to check the ability of one of the *Bacillus* strains, immobilized in polyacrylamide gel for the production of antibiotics and to optimize different physical and chemical parameters for antibiotic production.

MATERIALS AND METHODS

Source of reagents, chemicals and culture media

Reagents and chemicals (used in preparation of synthetic medium, immobilization and for morphological and biochemical identification), culture media (Nutrient agar and Tryptic soy broth) etc used in the present study are from Sigma (St Louis/USA) Oxoid (Hampshire/England) and Merck (Darmstadt/Germany).

Isolation, maintenance and cultural conditions

In the present study soil sprinkle technique was used to isolate antibiotic producing bacilli. For this purpose about 20-30 particles of soil from different locations of Quaid-i-Azam University, Islamabad, Pakistan were sprinkled on the surface of nutrient agar plates seeded with the test organism *Micrococcus luteus* (ATCC # 10240). The plates were incubated at 30°C for 24 hours. Antibiotic activity was checked by zone of inhibition, surrounding a colony. Different colonies having zones of inhibition were picked and streaked on separate nutrient agar plates to get pure cultures (Bushra *et al.,* 2007). These isolates were used as the source of antibiotic producing microbes. All strains were stored at 4°C and subcultured periodically. A total of five isolates were found to be producing zone of inhibition, out of which, one isolate showing a maximum inhibition zone was selected for further studies.

Identification of antibiotic producing soil bacilli

Isolated strain was identified morphologically (shape, Gram staining, spore staining, spore shape, sporangium dilatation and motility) and biochemically (anaerobic growth, growth in 5% NaCl, growth in 7% NaCl, growth in 10% NaCl, growth at pH 5.7, gelatin hydrolysis, indole production, citrate utilization, tyrosine utilization, VP-pH, VP-Acetoin, arabinose utilization, mannitol utilization, xylose utilization, methyl red-voges proskauer (MR-VP), oxidase production, catalase production, starch hydrolysis, nitrate reduction, casein hydrolysis, gas production from glucose, lecithinase production and production of SO_2) according to the Bergey's Manual of Determinative Bacteriology (Bergey and Holt, 1994)

Production medium and seed culturing (inoculum preparation)

Synthetic medium (L-Glutamic acid 5.0 g/L, KH_2PO_4 0.5 g/L, K_2HPO_4 0.5 g/L, $MgSO_4.7\ H_2O$ 0.2 g/L, $MnSO_4.\ H_2O$ 0.01 g/L, NaCl 0.01 g/L, $FeSO_4.7\ H_2O$ 0.01 g/L, $CuSO_4.7\ H_2O$ 0.01 g/L, $CaCl_2.2\ H_2O$ 0.015 g/L) was used as production medium. After sterilization of synthetic media concentrated glucose solution previously sterilized by 0.2 µm pore size filter paper, was added to give a final concentration of 1% in the medium. Seventy two hours old inoculum prepared in Tryptic Soy Broth (pH 7.3) was used at concentration of 10% (v/v). Inoculum was added to the production medium and incubated for 24 hours (Bushra *et al.,* 2007).

Immobilization in polyacrylamide gel

Whole cells of *Bacillus* strain were immobilized in polyacrylamide gel. The cells were collected by centrifugation at 10,000 rpm for 30 minutes and washed twice in sterilized saline. Washed cells were suspended in 3 ml saline and mixed with 6 ml of 20% polyacrylamide stock solution (18.2 gm acrylamide and 1.8 gm N, N-

methylene-bis-acrylamide dissolved in 50 ml distilled water, and diluted with distilled water to a final volume of 100 ml) and 100 µl ammonium persulphate (10%). 10 µl of N, N, N`, N` tetra methyl ethylenediamine (TEMED) was added to the mixture and polymerization was allowed to proceed for 20 minutes in an ice bath. Immobilized whole cells were cut into small blocks (8 to 27 mm^3) with a sterile knife blade, and washed thoroughly with sterilized saline (Yasushi *et al.,* 1979).

Antibiotic production and antimicrobial activity

Shake flask fermentation method was used for antibiotic production. Washed blocks were added aseptically in a 250 ml flask containing 50 ml of production medium pH 8 and the sample (cell free supernatant) was drawn at 0 hour (before incubation). The medium was then incubated in an orbital shaker (150 rpm) for 4 hours at 30 ºC and again sample was drawn. Finally the antimicrobial activity was determined by agar well diffusion assay (Awais *et al.,* 2008) using *Micrococcus luteus* as test microorganism and nutrient agar as an assay medium.

Optimization of cultural parameters

Incubation period (0 to 20 hours), initial pH of the production medium (6 to 9) and glucose concentration (1 to 5%) was optimized for maximum production of antibiotics by immobilized whole cells of *B. subtilis.* Immobilized whole cells of *B. subtilis* were incubated at 30 ^oC in an orbital shaker at 150 rpm and sample was drawn at 0 hour and then after every 4 hours, from 4 hours to 20 hours in case of time optimization, and at 0 hour and after 4 hours of incubation for pH and glucose optimization. Antimicrobial activity was determined through agar well diffusion assay (Awais *et al.,* 2008).

Statistical analysis

Simple statistics were calculated on the data obtained at different time periods, pH, glucose concentrations and zone of inhibition. The t-test to compare the means was calculated at $p<0.05$. Error bars obtained using standard deviation were also been used in bar charts at 5% level of probability.

RESULTS

Antibiotic producing soil bacilli

By using soil sprinkle technique five different colonies having zones of inhibition were picked and streaked on separate nutrient agar plates to get pure cultures. Out of all strains the one showing maximum inhibition zone was selected for optimization. Morphological and biochemical tests given in Table I indicated that the most probable identity of the isolate was *B. subtilis*.

Table I.- Morphological and biochemical tests for the identification of *Bacillus subtilis.*

Test	Morphological	Biochemical
Grams staining	+	
Shape	Cocco bacilli	
Spore formation	+	
Spore shape	Cylindrical	
Sporangium dilatation	-	
Motility	+	
Anaerobic growth		-
Growth in 5% NaCl		+
Growth in 7% NaCl		+
Growth in 10% NaCl		+
Growth at pH 5.7		+
Gelatin		+
Indole production		-
Citrate utilization		+
Tyrosine		-
VP-pH		<6
VP-Acetoin		+
Arabinose		+
Mannitol		+
Xylose		+
Methyl red (MR)		-
Voges-Proskauer (VP)		+
Oxidase		+
Catalase		+
Starch hydrolysis		+
Nitrate reduction		+
Casein hydrolysis		+
Gas production from glucose		-
SO_2		-
Lecithinase		-

Immobilization and antimicrobial activity

Small pieces of polyacrylamide gel containing immobilized whole cells of *B. subtilis* were added to the production medium pH 8.0. The

activity of the sample drawn at 0 hour and after 4 hours of incubation using *M. luteus* as test organism was almost the same, that is 18 mm and 19 mm, respectively.

Optimum growth conditions

Incubation time

During optimization of various parameters it was observed that the maximum antibiotic production was obtained after 4 hours of incubation (19 mm) and the second best activity was obtained at 0 hour (18 mm). Although good amounts were also observed at 8, 12, 16 and 20 hours of incubation period, however after 4 hours of incubation the amount of antibiotics started declining as shown in Figures 1 and 2. Free cell synthesis of peptide antibiotics from 0 to 144 hours is given in Figure 7. By comparing Figures 1 and 7, it is quite evident that higher quantities of antibiotics were produced in less time by immobilized cells of *B. subtilis*.

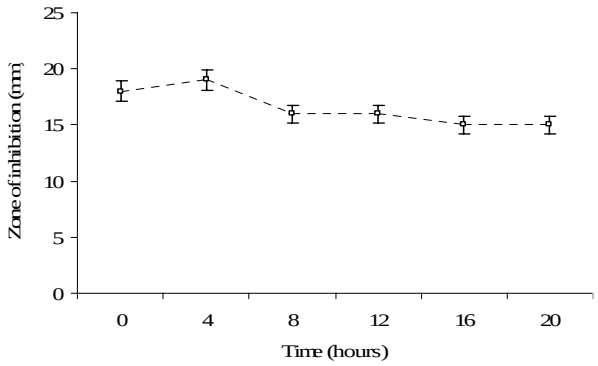

Fig. 1. Optimization of incubation time for the maximum production of antibiotics from *B. subtilis* against *M. luteus* in terms of zone of inhibition from 0 to 20 hours. Highest activity (19mm) was observed at 4 hours of incubation. Note that there is a progressive decrease in antibiotic production from 4 to 20 hours.

Initial pH

Immobilized *Bacillus* species were incubated in production medium with pH 6, 7, 8 and 9 at 30°C in an orbital shaker incubator. Maximum antibiotic production was obtained at pH 8 and 9, and considerable amount of antibiotic was also produced at pH 6-7. At pH 6 a small zone of inhibition was achieved at 0 hour of incubation as shown in Figures 3 and 4. However, pH 8 was used for further optimization experiments.

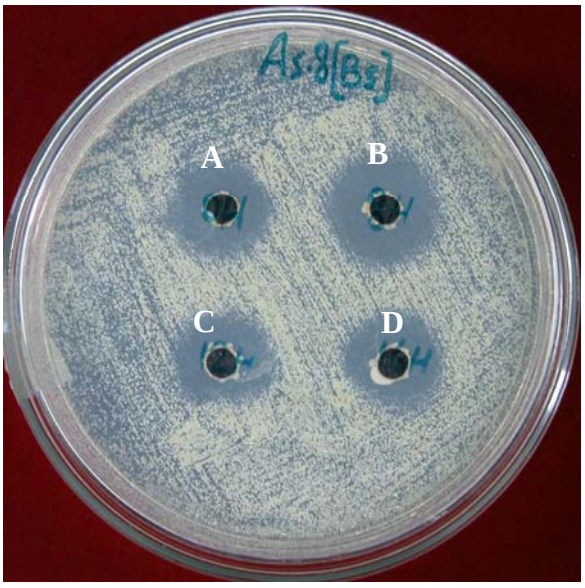

Fig. 2. Antimicrobial activity of *B. subtilis* at 0, 8, 12 and 16 hours of incubation, against *M. luteus*, as shown by clear zone of inhibition (mm) [A: 0, B: 8, C: 12, D: 16 hours]. Zone of inhibition directly related to the amount of antibiotic produced is decreasing with the passage of incubation time.

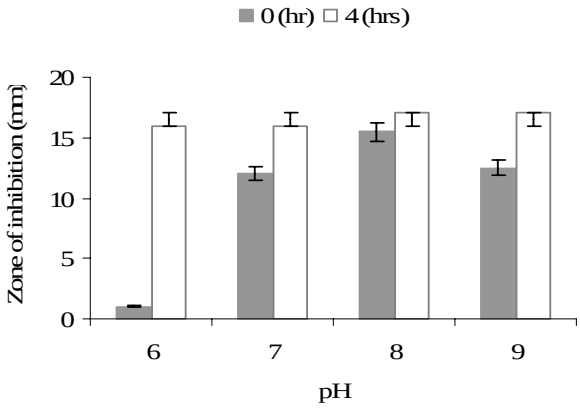

Fig. 3. Optimization of initial pH (6, 7, 8 and 9) for the maximum production of antibiotic from *B. subtilis* against *M. luteus* as indicated by zone of inhibition. Best activities were observed at pH 8.0 and 9.0.

Different glucose concentrations

Glucose concentration was varied from 1 to 5% in the production medium and checked for

maximum production of antibiotic. *B. subtilis* showed maximum zone of inhibition at 0 hour in the presence of 2% glucose. After 4 hours of incubation maximum zone of inhibitions were observed in the presence of 1% and 4% glucose, respectively as presented in Figures 5 and 6.

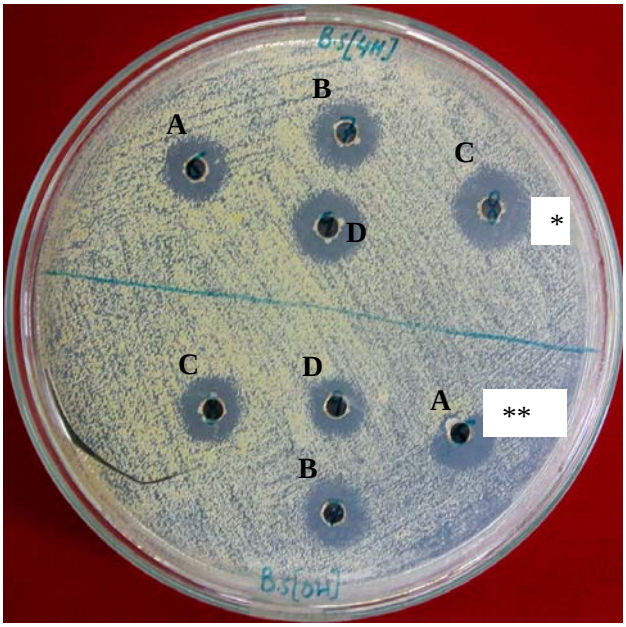

Fig. 4. Antimicrobial activity of *B. subtilis* at 0 and 4 hours against *M. luteus* at different pH, as shown by zone of inhibition (mm) [A, pH6; B, pH7, C, pH8; D, pH9].
* Zone of inhibition at 4 hours.
**Zone of inhibition at 0 hour.

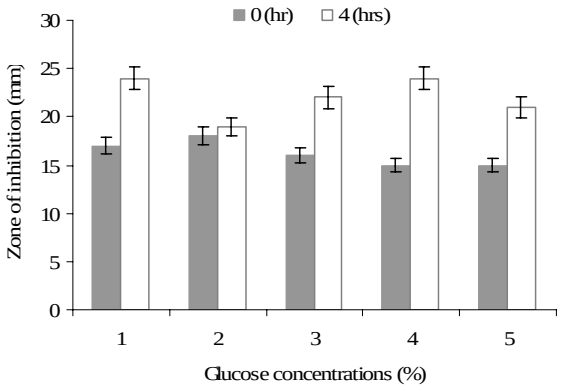

Fig. 5. Maximum production of antibiotic by *B. subtilis* against *M. luteus* at different glucose concentrations. *B. subtilis* showed maximum activity (24mm) after 4 hours of incubation in 1% and 4% glucose, respectively.

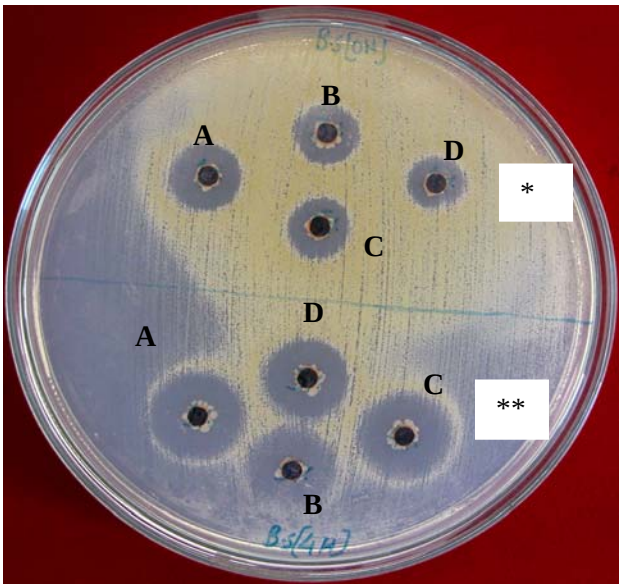

Fig. 6. Antimicrobial activity of *B. subtilis* at 0 and 4 hours of incubation against, *M. luteus* at different concentrations of glucose, as shown by zone of inhibition (mm) [A, 1%; B, 3%; C, 4%; D, 5%].
*Zone of inhibition at 0 hour.
** Zone of inhibition at 4 hours.

DISCUSSION

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 (Jensen and Wright, 1997; Talaro and Talaro, 1996). Rhizobacteria are present in the soil in an average of about 108 cells per gram (Stein, 2005). *B. subtilis* is also a rhizobacterium and an endospore forming bacteria (Sonenshein *et al*., 2001). The present study was carried out to evaluate the production of antibiotic from newly isolated *Bacillus* specie from soil by soil sprinkling method, which was identified as *B. subtilis* by performing various morphological and biochemical tests (Table I) according to Bergey's Manual of Determinative Bacteriology (Bergey and Holt, 1994). Similar experiments were carried out by Bushra *et al.* (2007) and Awais *et al.* (2008) for the isolation and

identification of antibiotic producing soil bacilli. Members of the genera *Bacillus*, *Streptomyces* and *Pseudomonas* are soil bacteria that produce a high proportion of agriculturally and medically important antibiotic (Sharga *et al.,* 2004; Yoshiko *et al.,* 1998). The potential of *B. subtilis* to produce antibiotics has been recognized for 50 years. Peptide antibiotics represent the predominant class (Pinchuk *et al.,* 2002). According to Sonenshein *et al*. (2001) several hundred wild- type *B. subtilis* strains have been collected, with the potential to produce more than two dozen antibiotics.

In the present study synthetic media containing all essential minerals, carbon and nitrogen sources was used as production media. It is because certain metal ions are required for the activity and proper functioning of polypeptide antibiotics, 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). Bushra *et al.* (2007) also used similar synthetic media and obtained considerably better activity as compared to that obtained by using nutrient broth as a production medium because nutrient broth was not fulfilling all the nutritional requirements necessary for antibiotic production.

In the past years much interest has been expressed in the use of microorganisms immobilized in solid supports (Kierstan and Buche, 1977; Chibata and Tosa, 1977). In immobilized state bacteria are active for a long time in the gel (Morikawa *et al.,* 1979). Several antibiotics have been reported to be produced by immobilized cells. Erythromycin has been produced *by Saccharopolyspora erythraea* immobilized in 2% (w/v) calcium alginate and showed higher titers of erythromycin (2.3 times more than that of the control) (Hamedi *et al.,* 2005). *Penicillium chrysogenum* have been immobilized in polyacrylamide gel and used for the production of penicillin (Morikawa *et al.,* 1979). In the present study *B. subtilis* also produced antimicrobial metabolites as evident from zone of inhibition (18 mm and 19 mm) against *M. luteus* during initial screening. Inhibitions of various organisms by *Bacillus* species have been reported by different scientists. Azevedo *et al*. (1993) isolated a strain of *B. subtilis* C126 from sugar cane fermentation, which produced a polypeptide antibiotic, bacitracin, which inhibited the growth of *Micrococcus flavus*. A *B. licheniformis* strain, 189, isolated from a hot spring environment in the Azores, Portugal, was found to strongly inhibit growth of Gram-positive bacteria by producing peptide antibiotic (Mendo *et al*., 2004).

In the present study the rate of antibiotic production by immobilized whole cells was slightly higher than that of the free cells (Comparison of Fig. 1 with Fig. 7 clearly indicates the difference). Similar results have been reported by Morikawa *et al.* (1979). He reported that bacitracin productivity

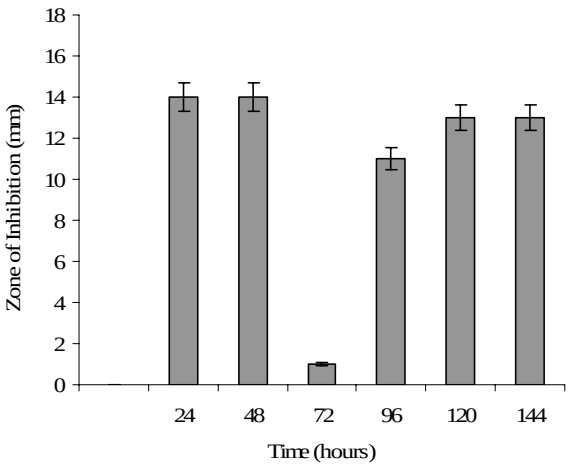

Fig. 7. Antimicrobial activity of free cells of *B. subtilis* from 0 to 144 hours of incubation, against *M. luteus*, in terms of zone of inhibition (mm).

by the immobilized whole cell containing air bubbled reactor was higher than that by a conventional continuous and batch fermentation process by high dilution rates. Srinivasulu *et al.* (2003) have studied the effect of *Streptomyces marinensis* NUV-5 cells immobilized in calcium alginate for the production of neomycin and reported an enhanced antibiotic productivity of 32% was achieved with immobilized cells over the conventional free-cell fermentation. In the present

study production of antibiotics also decreased with successive utilization of immobilized cells (Figs. 1, 2). Moreover during free cell synthesis of peptide antibiotics, maximum activity (14mm) was seen during 24 to 48 hours of incubation, which abruptly decreased at 72 hours and then again good activities (13mm) were seen at 120 and 144 hours of incubation (Fig. 7). It has been reported by Haavik (1975) that bacitracin production by *B. licheniformis* ATCC 14580 was observed only during the phase of rapid growth. The present study showed similar observation where maximum production was found during 24 to 48 hours of incubation, the phase of rapid growth for the *Bacillus* sp. Whereas, according to Egorov *et al*. (1986) maximum efficiency of the bacitracin synthesis in case of *B. licheniformis* coincides with the end of the exponential growth phase and the onset of sporification. *B. subtilis* is an endospore forming bacterium and during incubation from 0 to 48 hours, bacterium maximally utilizes the nutrients available in the surrounding synthetic medium and produces maximum amount of antimicrobial metabolites. During the next 24 hours, *B. subtilis* starts producing endospore having very limited metabolic activities including the formation of antimicrobial metabolites; it is, therefore, a very small zone of inhibition is observed at 72 hours of incubation. Later on (at 120 and 144 hours) the endospores germinate again, resume their metabolic activity and start producing good amounts of antibiotics.

In the present study good activities were obtained at all the pH values but maximum activity was observed at alkaline pH of 8 and 9 (Figs. 3, 4). It has earlier been reported by Anker *et al.* (1947) that pH of 7.8-8 gave maximum production of bacitracin. Changes in external pH affect many cellular processes such as the regulation of the biosynthesis of secondary metabolites (Solé *et al.*, 1994, 1997). Haavik (1975) reported that bacitracin production by *B. subtilis* is pH dependent. It has been reported by Yousaf (1997) that optimum bacitracin yield from *B. licheniformis* was obtained with initial pH of 7.0.

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 (Datta and Kothary, 1993; Espeso *et al.,* 1993). The effect of glucose concentration on the production of antibiotic in immobilized state was studied. In immobilized state *B. subtilis* produced maximum zone of inhibition (24 mm) at 4 hours in 1 and 4% glucoses, respectively (Figs. 5, 6). According to a study penicillin produced by the immobilized *P. chrysogenum* in the presence of glucose was 17% of that produced by washed mycelium. The activity of the immobilized mycelium increased initially and decreased gradually with repeated use (Morikawa *et al.,* 1979). Better neomycin production was achieved with 3% wt/vol maltose by *Streptomyces marinensis* NUV-5 cells immobilized in calcium alginate (Srinivasulu *et al.,* 2003). Carbohydrates in the form of sugars were employed for the production of bacitracin by *B. licheniformis*. Among sugars glucose gave the maximum yield of antibiotic and gave maximum yield at 0.5% (w/v) concentration after its complete consumption (Yousaf, 1997).

Some researchers have shown results, which are not in agreement with our study. Production of nisin by the cells of *Streptococcus lactis* immobilized in polyacrylamide gel was studied by Egorov *et al.* (1978) and it was found that the amount of the antibiotic was 2-3 times lower than in case of using free cells. In a study by Morikawa *et al.* (1979) leakage of the bacteria from the gel was observed during fermentation. But in our study, there was no leakage of cells from the gel as evident by determination of optical density (OD) of the fermentation medium during culturing.

Bacillus species are supposed 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). Carriers for immobilization of the bacteria which has good mechanical properties and good diffusibility of substrates must also be considered in any further studies. Recent advances in the molecular genetics of antibiotic biosynthesis have opened new perspectives for antibiotic production. By cloning and amplifying the gene coding for some key enzymes in the biosynthetic pathways of bacitracin, it is possible to enhance the yield of bacitracin (Morikawa *et al.,* 1979).

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 *B. subtilis* was the best antibiotic-producing strain among the tested organisms. The optimum pH for antibiotic production by *B. subtilis* was 8, although good activity was also achieved at pH 6-9. The maximum antibiotic activity by *B. subtilis* was achieved at 0 hour and after 4 hours of incubation. Activity was different at 0 and 4 hours, at various glucose concentrations. *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.

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