Pak. J. Bot., 41(3): 1499-1510, 2009.

PRODUCTION OF ANTIBACTERIAL COMPOUNDS BY FREE AND IMMOBILIZED *BACILLUS PUMILUS* SAF1

FARIHA HASAN, SAMIULLAH KHAN, AAMER ALI SHAH* AND ABDUL HAMEED

Department of Microbiology,
Quaid-i-Azam University, Islamabad, Pakistan

Abstract

In the present study, *Bacillus pumilus* SAF1, capable of producing antimicrobial compounds, was indigenously isolated from soil. Antimicrobial substances were found to be produced by *Bacillus pumilus* SAF1 against *Micrococcus luteus* (ATCC # 10240) and *Staphylococcus aureus* (ATCC # 6538) used as test organisms. Maximum production of antimicrobial compounds by *Bacillus pumilus* SAF1, was observed at pH 7, 3% glucose and after 48 hours of incubation at 30ºC, on the basis of zones of inhibition against *Micrococcus luteus* (ATCC # 10240) and *Staphylococcus aureus* (ATCC # 6538). Whole cells of *Bacillus pumilus* SAF1 were immobilized in polyacrylamide gel, sodium alginate and agar-agar, and checked for antibiotic production by Agar Diffusion Assay, against *Micrococcus luteus* (ATCC # 10240) and *Staphylococcus aureus* (ATCC # 6538), and various parameters [pH (6-9), incubation period (0-72 hours) and glucose concentration (1-5%)] were optimized for maximum production of antibiotics. Maximum activity in the form of zone of inhibition (32mm) was observed at pH 7, 3% glucose, and after 72 hours of incubation in polyacrylamide gel. As a whole, the antimicrobial activity was higher in sodium alginate and agar-agar as compared to free cells fermentation. As the activity of antibiotic production increased in case of immobilized cells, so it can be concluded that in the present study, immobilization proved to be a better process, as compared to production of antibiotic from free cell.

Introduction

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 been derived from a relatively small group of microorganisms belonging to the genera *Penicillium, Streptomyces, Cephalosporium, Micromonospora* and *Bacillus*. More than 5000 different antibiotics have been isolated from cultures of bacteria, fungi and plant cells, 60% of them are contributed by the genus *Streptomyces* (Park *et al*., 1998).

In pharmaceutical industry several peptide antibiotics of importance are produced by *Bacillus* species such as bacitracin, polymyxin, gramicidin, tyrocidine, subtilin, bacilysin etc. Majority of the antibiotics from *Bacillus* sp., are low molecular weight peptides, produced *via* the nonribosomal biosynthetic pathway, which involves specific enzymes called peptide synthetases. These peptides possess a varied range of remarkable biological activities, including antimicrobial, antiviral and antitumoral activities (Cane *et al*., 1998). More than 500 peptide antibiotics have been described in the past half-century. These peptide antibiotics differ widely in their biochemical properties (amino acid composition, length and secondary structure). However, they all play essential roles in non-specific host defenses by preventing or limiting infections by their ability to selectively recognize potential pathogens (Smet & Contreras, 2005). Most peptides exert

*Corresponding author E-mail: alishah_75@yahoo.com; Tel. No. +92-51-90643065; Fax No. +92-51-9219888

their antifungal or antibacterial effects by interacting with and destabilizing the microbial membrane, leading to cell death. However, different modes of action are proposed for several peptides, including inhibiting synthesis of specific membrane proteins, or stress proteins, arrest of DNA synthesis, breakage of single-strand DNA, interaction with DNA (Park *et al*., 1998), and production of hydrogen peroxide. Antimicrobial peptides can also act by triggering apoptosis in eukaryotic cells or autolysis in bacterial targets (Chitnis *et al*., 1993).

Now a day's microbial cell are immobilized to produce a number of products like enzymes and antibiotics etc. Immobilization of microbial cells in biological processes can occur either as a natural phenomenon or through artificial process. While the attached cells in natural habitat exhibit significant growth, the artificially immobilized cells are allowed restricted growth. During the last 20–25 years, the cell immobilization technology, with its origins in enzyme immobilization has attracted the attention of several research groups. This novel process eliminates most of the constraints faced with the free-cell systems, such as low cell density, nutritional limitations and batch-mode operations with high down times. The remarkable advantage of this new system is the freedom it has to determine the cell density prior to fermentation. It also facilitates operation of microbial fermentation on continuous mode without cell washout. The whole-cell immobilization process decouples microbial growth from cellular synthesis of favored compounds. The ease of conversion of batch processes into a continuous mode and maintenance of high cell density without washout conditions even at very high dilution rates, are few of the many advantages of immobilized cell systems. There is considerable evidence to indicate that the bound-cell systems are far more tolerant to perturbations in the reaction environment and similarly less susceptible to toxic substances present in the liquid medium. The recent reports on higher retention of plasmid-bearing cells have further extended the scope of whole-cell immobilization to recombinant product formation. Another important advantage of immobilization, particularly in the case of plant cells, is the stimulation of secondary metabolite formation and elevated excretion of intracellular metabolites (http://www.ias.ac.in/currsci/jul10/articles17.htm).

Immobilized cells have been defined as cells that are entrapped within or associated with an insoluble matrix. The application of immobilized cells to study microbial processes is one of the main trends in modern biotechnology. Immobilization techniques mostly implied for the production of secondary metabolites are cell entrapment and cell adsorption on solid support (Asanza *et al*., 1997). Cell immobilization shows many operational and economic advantages such as prolong metabolic activities, reuse of the biocatalyst, increase of cell concentration in preventing washing out of cells at high flow rates (Gautam *et al*., 2002).

The present study aimed to study the antimicrobial producing capabilities of *Bacillus pumilus* SF1 in both free and immobilized environments and also optimizing different culture conditions for maximum production.

Material and Methods

Isolation and identification of antimicrobial compound producing *Bacillus* species: The antibiotic producing *Bacillus* species were isolated from soil samples collected from Quaid-i-Azam University, Islamabad, Pakistan. Lawn of susceptible test organisms; *Micrococcus luteus* (ATCC # 10240) and *Staphylococcus aureus* (ATCC # 6538) was made on nutrient agar plates and soil was sprinkled on the lawn. Plates were incubated at 30ºC for 24 hours. The microbial colonies with clear zones of inhibition around them

were selected as antibiotic producing isolates and identified on the basis of their morphological and biochemical characteristics according to Bergey's Manual of Determinative Bacteriology (Holt, 1993).

Inoculum preparation: The inoculum *of Bacillus* specie, identified as *Bacillus pumilus SAF1,* selected on the basis of maximum activity against the test organisms, was prepared in nutrient broth by incubating at 30˚C for 48 hours in an orbital shaker at 120 rpm.

Production of antimicrobial compounds: About 100 ml of the sterilized synthetic medium (g/L); L-glutamic acid 5.0; KH_2PO_4 0.5; K_2HPO_4 0.5; $MgSO_4.7H_2O$ 0.2; $MnSO_4.H_2O$ 0.01; NaCl 0.01; $FeSO_4.7H_2O$ 0.01; $CuSO_4.7H_2O$ 0.01; $CaCl.2H_2O$ 0.015; Glucose 10; and pH 7), was taken in 250 ml flask. Inoculum (10%) was added to the flask and incubated at 30˚C in orbital shaker at 120 rpm. Samples were taken after every 24 hours, centrifuged to get cell free supernatants, sterilized through 0.2 µm filter paper and stored at -20ºC for further use.

Agar diffusion assay: Agar well diffusion method was used to check the cultures for the production of antimicrobial metabolites (Sen *et al*., 1995). Twenty-four hours fresh cultures of *M. luteus* (ATCC # 10240) and *S. aureus* (ATCC # 6538) were diluted with pre-sterilized normal saline and the turbidity of the cultures was adjusted with 0.5 MacFarland. A sterilized cotton swab was dipped in the diluted cultures and lawns were prepared over the agar surface. Wells were made in the inoculated plates using sterile cork borer. About 80 µl cell free supernatants were added in the wells and the plates were incubated at 37ºC for 24 hours. After 24 hours, the zones of inhibition were observed. The diameter of the zone of inhibition was measured in mm.

Optimization of various parameters for maximum antibiotic production: Incubation period (0-72 hrs.), initial pH of the medium (6-9) and glucose concentration (1-5%) was optimized for maximum production of antibiotics by *B. pumilus* SAF1.

Immobilization of *Bacillus pumilus* SAF1 in polyacrylamide: *B. pumilus* SAF1 culture was refreshed on nutrient agar. About 5 ml medium (nutrient broth) was taken in test tube and autoclaved. After sterility checking the broth tube was inoculated with *B. pumilus* SAF1 and incubated at 30ºC for 24 hours in orbital shaker at 120 rpm. Nutrient broth flask 45 ml was prepared and autoclaved at 121ºC. Next day the 5 ml inoculum from the test tube was transferred into 45 ml medium in the flask and incubated at 30ºC for 24 hours in orbital shaker at 120 rpm. From this flask 3 ml of inoculum was 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 further 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 blade and washed thoroughly with sterilized distilled water twice and then added aseptically to 100 ml nutrient broth in 250 ml flask and was incubated at 30ºC for 48 hours in orbital shaker at 120 rpm. These blocks were washed with distilled water and added aseptically in a 250 ml flask containing 50 ml synthetic media having pH 7 and incubated at 30ºC for 24 hours in an orbital shaker at 120 rpm. After 24 hours these blocks were washed with distilled water

and again added to 50 ml synthetic medium. The effect of different pH ranges from 6-9 and glucose concentration 1-5% and incubation time 0-72 hours was checked. After each sample the cubes were washed with distilled water and the medium was replaced with fresh one. The samples were filtered through 0.2µm membrane filter. Finally the antimicrobial activity was determined by agar diffusion assay using *M. luteus* (ATCC # 10240) and *S. aureus* (ATCC # 6538) as test organisms.

Immobilization of *Bacillus pumilus* SAF1 in Sodium alginate: About 45 ml of nutrient broth was inoculated with 5 ml of *B. pumilus* SAF1 inoculum, and then incubated at 30ºC for 24 hours in an orbital shaker at 120 rpm. Out of this 50 ml of culture, 3 ml of inoculum was mixed with 100 ml (3%) Sodium alginate slurry. The slurry was taken into a sterile syringe and added drop wise into 0.2 M $CaCl_2$ solution from 5 cm height to get Sodium alginate beads. The beads were washed with sterile distilled water twice, then added to 100 ml nutrient broth in 250 ml flask and was incubated at 30ºC for 48 hours in an orbital shaker at 120 rpm. The beads were washed with distilled water and added aseptically to 50 ml synthetic medium having pH 7 in a 250 ml flask, and incubated at 30ºC for 24 hours in orbital shaker at 120 rpm. After 24 hours these beads were washed with distilled water and again added aseptically to 50 ml synthetic medium. The effect of different pH ranges from 6-9 and glucose concentration 1-5% and incubation time 0-72 hours was checked. The samples were filtered through 0.2µm membrane filter. Finally the antimicrobial activity was determined by agar diffusion assay using *M. luteus* (ATCC # 10240) and *S. aureus* (ATCC # 6538) as test organisms.

Immobilization of *Bacillus pumilus* SAF1 in agar-agar: About 45 ml of nutrient broth was inoculated with 5 ml of *B. pumilus* SAF1 inoculum and then incubated at 30ºC for 24 hours in orbital shaker at 120 rpm. From this flask 3 ml of inoculum was mixed with sterilized 18 ml agar-agar solution prepared in 0.9% Sodium chloride solution to get final concentration of 2%. The molten agar-agar maintained at 40°C, shaken well for few seconds, poured into sterile flat bottom 4-inch-diameter Petri plates and allowed to solidify. The solidified agar block was cut into equal size cubes (4 mm^3). The cubes were washed with sterile distilled water twice and were added aseptically to 100 ml nutrient broth in a 250 ml flask, incubated at 30ºC for 48 hours in orbital shaker at 120 rpm. These cubes were washed with distilled water and added aseptically in a 250 ml flask containing 50 ml synthetic medium having pH 7 and incubated at 30ºC for 24 hours in an orbital shaker at 120 rpm. After 24 hours these cubes were washed with distilled water and again added aseptically to 50 ml synthetic medium in a 250 ml flask and the sample was drawn at 0 hours. The medium containing cubes was then incubated in orbital shaker (120 rpm) at 30ºC and the sample was drawn from 4 to 72 hours at different time intervals. After each sample the cubes were washed with distilled water and the medium was replaced with fresh one. The samples were filtered through 0.2µm membrane filter. Finally the antimicrobial activity was determined by agar diffusion assay using *M. luteus* (ATCC # 10240) and *S. aureus* (ATCC # 6538) as test organisms.

Results

Identification of *Bacillus* species: *Bacillus* specie, isolated from soil which showed zone of inhibition against the test organisms, *M. luteus* (ATCC # 10240) and *S. aureus* (ATCC # 6538), was identified as *Bacillus pumilus* SAF1.

Antibiotic production by free cells of *Bacillus pumilus* SAF1: Samples drawn during batch fermentations were subjected to agar diffusion assay, using *M. luteus* (ATCC # 10240) and *S. aureus* (ATCC # 6538) as test organisms. Antimicrobial activity was measured in terms of zone of inhibition (mm). Maximum production of antimicrobial compounds by *B. pumilus* SAF1, was observed at pH 7, 3% glucose, after 48 hours against both *M. luteus* (ATCC # 10240) and *S. aureus* (ATCC # 6538) (Figs. 1 & 2).

Antibiotic production by *Bacillus pumilus* SAF1 immobilized in polyacrylamide gel: Whole cells of *B. pumilus* SAF1 were immobilized in polyacrylamide gel and the antimicrobial activity was determined by agar diffusion assay, using *M. luteus* (ATCC # 10240) and *S. aureus* (ATCC # 6538) as test organisms. Maximum antibiotic production by *B. pumilus* SAF1 immobilized in polyacrylamide gel was observed at pH 7, 3% glucose, after 72 hours against *M. luteus* (ATCC # 10240) and *S. aureus* (ATCC # 6538) (Figs. 3 & 4).

Antibiotic production by *Bacillus pumilus* SAF1 immobilized in sodium alginate: Maximum antimicrobial production by *B. pumilus* SAF1 immobilized in Sodium alginate was observed at pH 7, at 3% glucose, after 24 hours against *M. luteus* (ATCC # 10240) and *S. aureus* (ATCC # 6538) (Figs. 5 & 6).

Antibiotic production by *Bacillus pumilus* SAF1 immobilized in agar-agar: Maximum antibiotics production by *B. pumilus* SAF1 immobilized in agar-agar was observed at pH 7, 4% glucose after 72 hours, against *M. luteus* (ATCC # 10240) while at 3% glucose concentration *S. aureus* (ATCC # 6538), maximum zone of inhibition was observed after 48 hrs. at 72 hours (27 mm) and (24 mm) at 48 hours (Figs. 7 & 8).

Discussion

There are many species of the genus *Bacillus* which can produce a wide variety of antibiotics including bacitracin, polymyxin, colistin etc. Several bacitracins have been characterized; the bacitracin A is the dominant commercial product (Schallmey *et al.,* 2004). *Bacillus* antibiotics are generally produced at the early stages of sporulation process demonstrated the transfer of the bacitracin biosynthetic gene cluster from *B. licheniformis* to the engineered host *B. subtilis* and the biosynthesis of bacitracin in high levels.

The present research work was carried out to optimize the conditions for the production of bioactive microbial metabolites by *Bacillus pumilus* SAF1 in free as well as in immobilized state. The *Bacillus* species were isolated from soil and identified according to Bergey's Manual of Determinative Bacteriology (Holt, 1993).

In the present study the antibacterial activity of the organism was tested against different species of organisms but they exhibited better activity against Gram-positive *M. luteus* (ATCC # 10240) and *S. aureus* (ATCC # 6538). Inhibition of various organisms has been reported by different scientists, Azevedo *et al*., (1993) isolated a strain of *Bacillus subtilis* C126 from sugar cane fermentation, which produced a polypeptide antibiotic bacitracin which inhibited the growth of *Micrococcus flavus*. A *B. licheniformis* strain189 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).

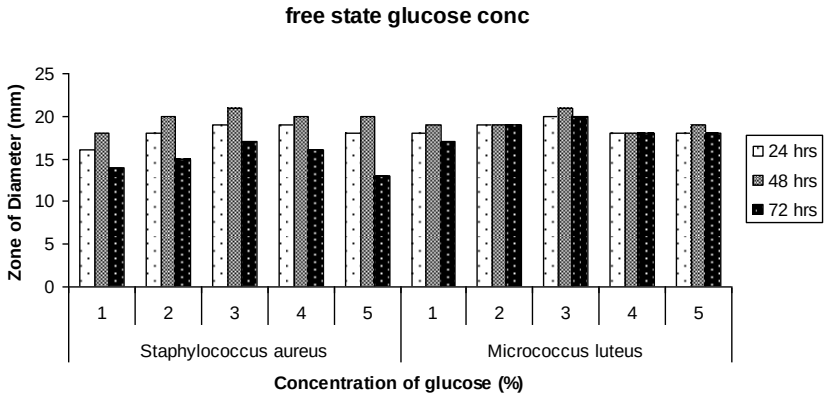

Fig. 1. Maximum antibiotic produced by free cells of *Bacillus pumilus* SAF1 at different concentrations of glucose.

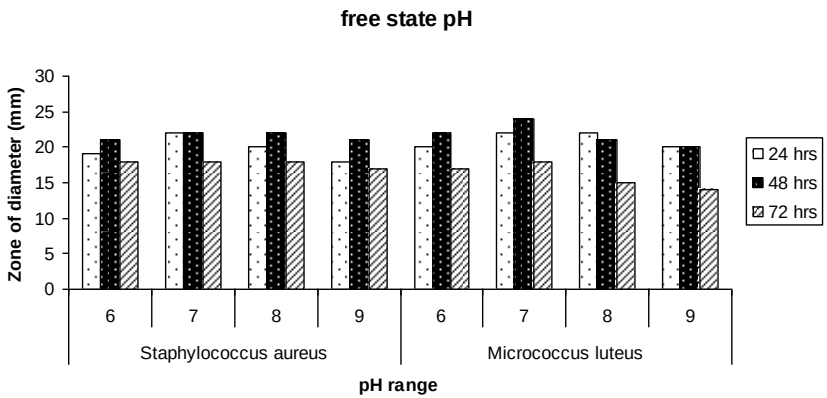

Fig. 2. Maximum antibiotic produced by free cells of *Bacillus pumilus* SAF1 at different pH.

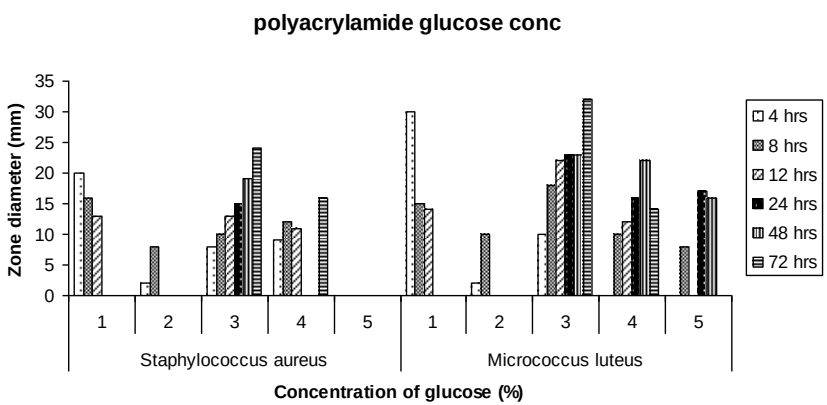

Fig. 3. Maximum antibiotic produced by *Bacillus pumilus* SAF1 immobilized in polyacrylamide gel at different concentrations of glucose.

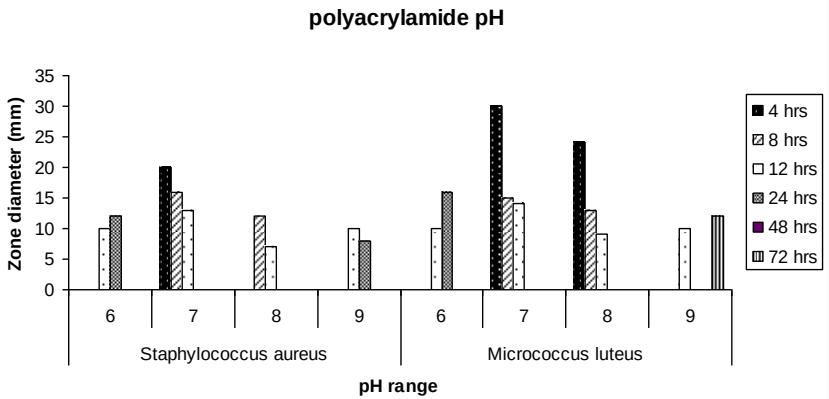

Fig. 4. Maximum antibiotic produced by *Bacillus pumilus* SAF1 immobilized in polyacrylamide gel at different pH.

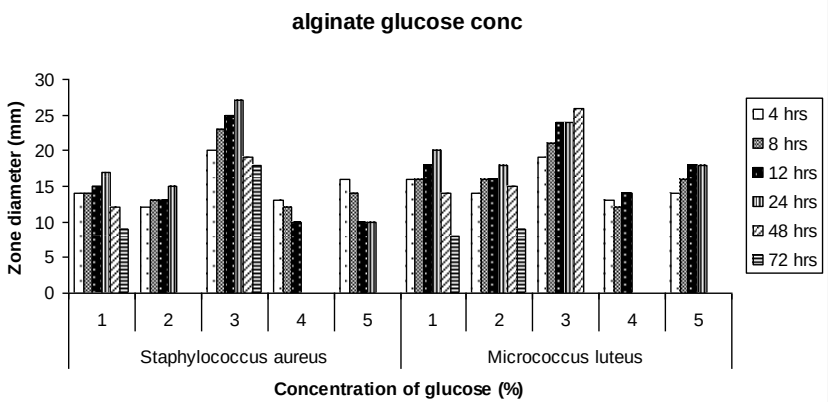

Fig. 5. Maximum antibiotic produced by *Bacillus pumilus* SAF1 immobilized in sodium alginate at different concentrations of glucose.

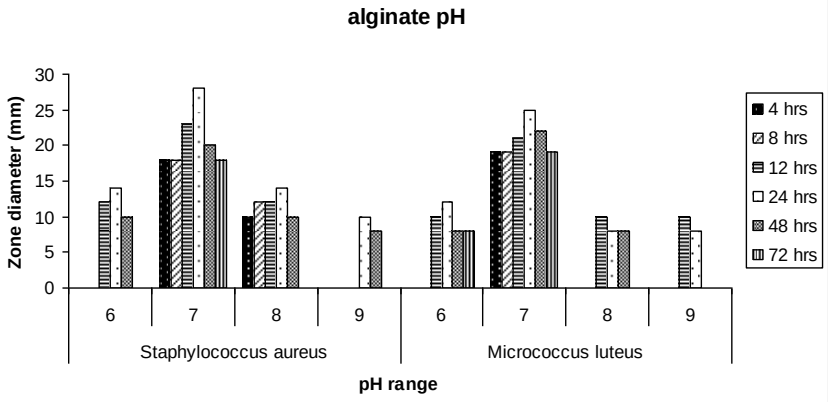

Fig. 6. Maximum antibiotic produced by *Bacillus pumilus* SAF1 immobilized in sodium alginate at different pH.

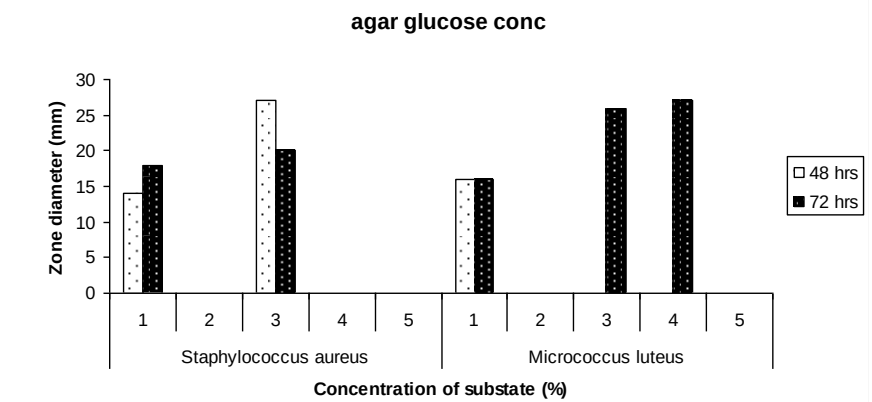

Fig. 7. Maximum antibiotic produced by *Bacillus pumilus* SAF1 immobilized in agar-agar at different concentrations of glucose.

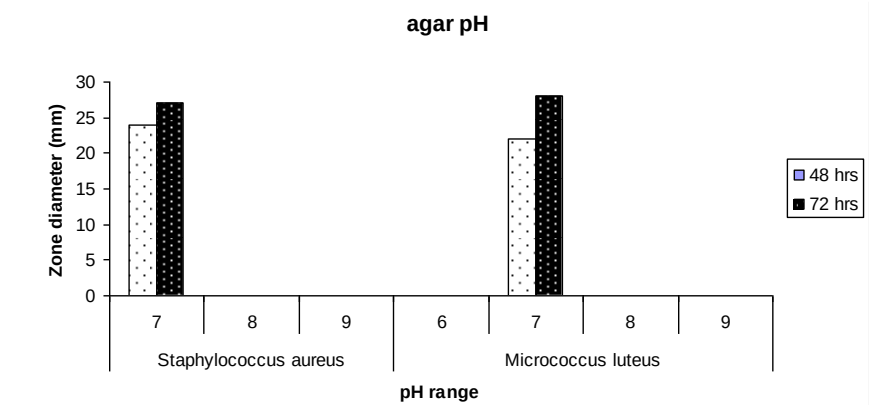

Fig. 8. Maximum antibiotic produced by *Bacillus pumilus* SAF1 immobilized in agar-agar at different pH.

In the search for antiinfective produced by *Bacillus* species, especially *Bacillus cereus*, *Bacillus subtilis* and *Bacillus licheniformis* having several antifungal compounds, mainly peptides, have also been described (Lebbadi *et al*., 1994). Bottone & Peluso (2003) have reported a compound produced by *Bacillus pumilus* (MSH) that inhibits *Mucoraceae* and *Aspergillus* species.

Initial screening, in the present study, showed that maximum inhibition was observed by the strain at 30ºC against *M. luteus* (ATCC # 10240) and *S. aureus* (ATCC # 6538). Therefore, further studies were carried out at this temperature. Early studies by Lebbadi *et al*., (1994) have shown that maximum titers of bacitracin were obtained at 37ºC after 3-5 days of incubation whereas, at 35ºC maximum titer was usually attained after 120 hours of incubation.

In the present study production of antibiotics was studied at different time periods i.e., 0, 24, 48 and 72 hours of incubation. It was found that maximum zone of inhibition (about 24 mm) was produced by *Bacillus pumilus* SAF1 (cell free extract) against *M. luteus* (ATCC # 10240) and (about 22 mm) against *S. aureus* (ATCC # 6538).

It has been reported by Haavik (1975) that bacitracin production by *Bacillus licheniformis* (ATCC # 14580) was observed only during the phase of rapid growth. The present study showed similar observation where maximum production was found during 48-72 hour incubation, the phase of rapid growth for the *Bacillus* spp. 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 at the onset of spore formation. In submerged fermentation, 20 hours old vegetative inoculum gave the maximum yield of bacitracin by *B. licheniformis* (Yousaf, 1997).

In the present study antibiotic producing bacteria *B. pumilus* SAF1 was immobilized in polyacrylamide gel, Sodium alginate and agar-agar and used for the production of antibiotic. Various parameters like, incubation period, pH, and glucose concentrations were optimized and compared with the production of antibiotic under same parameters.

Effect of pH was studied by adjusting the initial pH range (6, 7, 8, and 9) of the production medium. It is evident from the results that *Bacillus pumilus* SAF1 (cell free extract) showed increased inhibition (30 mm) after 72 hours of incubation at 7 pH, against *M. luteus* and (32mm) against *S. aureus* when immobilized in polyacrylamide gel. It has earlier been reported by Anker *et al*., (1947) that pH of 7.8 and 8 gave maximum production of bacitracin. Whereas, Iglewski & Gerhardt (1978) isolated a strain of *Bacillus subtilis* with activity against *Proteus vulgaris* within the pH range of 5.7 to 6.8. It has been reported by Yousaf (1997) that optimum bacitracin yield from *B. licheniformis* was obtained with initial pH of 7.0. *Penicillium chrysogenum* have been immobilized in polyacrylamide gel and used for the production of Penicillin (Morikawa *et al*., 1979).

The effect of glucose concentration on the production of antibiotic in free as well as in immobilized state (polyacrylamide gel) was studied. Maximum zone of inhibition (32mm) was produced by *B. pumilus* against *M. luteus* at 72 hours in 3% glucose while a maximum zone (24mm) against *S. aureus* in 3% glucose at 72 hours. According to a study penicillin produced by the immobilized *Penicillium 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% w/v 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 antibiotics. Glucose also proved as best carbon source so it gave maximum yield at 0.5% (w/v) concentration after its complete consumption (Yousaf, 1997).

In the past many years, much interest has been expressed in the use of microorganisms immobilized on solid supports. In immobilized state bacteria are active for a long time in the gel (Morikawa *et al*., 1979). Romo & Perezmartinez (1997) reported the viability of microbial cells over a period of 18 months under entrapped conditions and it was considered as one of the potential applications. Several antibiotics have been reported to be produced by immobilized cells. *B. pumilus* SAF1 was immobilized in Sodium alginate and various parameters like, incubation period, pH and glucose concentrations were optimized and compared with the production of antibiotic under same parameters. It is evident from the results that *Bacillus pumilus* SAF1 (cell free extract) showed increased inhibition (28 mm) after 24 hours of incubation at pH 7, when tested against *M. luteus* and (25mm) against *S. aureus* as compared to cells immobilized in alginate.

The effect of glucose concentration on the production of antibiotic in free as well as in immobilized state (Sodium alginate) was studied. Maximum zone of inhibition (27mm) was produced by *B. pumilus* against *M. luteus* at 24 hours in 3% glucose while *B. pumilus* SAF1 produced a maximum zone (24mm) against *S. aureus* in 3% glucose at 24 hours. According to a study Penicillin produced by the immobilized *Penicillium 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).

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).

B. pumilus SAF1 was immobilized in agar-agar and various parameters like incubation period, pH, and glucose concentrations were optimized and compared with the production of antibiotic under same parameters. It is evident from the results that *B. pumilus* SAF1 (cell free extract) showed increased inhibition (28 mm) after 72 hours of incubation at 7 pH, when tested against *M. luteus* and (27mm) against *S. aureus.*

The effect of glucose concentration on the production of antibiotic in free as well as in immobilized state (agar-agar) was studied. Maximum zone of inhibition (27mm) was produced by *B. pumilus* SAF1 against *M. luteus* at 72 hours in 4% glucose while a maximum zone of (27mm) against *S. aureus* in 3% glucose at 48 hours.

Tonkova *et al*., (1994) have been successful to immobilize cells of *Bacillus licheniformis* 44MB82-G by entrapment in agar gel or by binding to formaldehyde-activated acrylonitrile/acrylamide membranes and use for the production of thermostable alpha-amylase. Highest titer of erythromycin (2.3 times more than that of the control) was achieved in medium containing 1 g agar/l (Hamedi *et al*., 2005).

Changes in external pH affect many cellular processes such as the regulation of the biosynthesis of secondary metabolites (Sole *et al*., 1997). 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 (Espeso *et al*., 1993). Haavik (1974) reported that 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.

In the present study the rate of antibiotics production by immobilized whole cells was slightly higher than that of the free cells. Similar results have been reported by Morikawa *et al*., (1979), who reported that bacitracin productivity 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.

Some of the researchers have shown results, which are not in line 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 bacteria from the gel was observed during fermentation. In our study, there was no leakage of cells from the gel as evident by determination of OD of the fermentation medium during culturing.

References

Asanza, M.L., E. Gontier, C. Bienaime, J.E. Nava Saudedo and J.N. Barotin. 1997. Response surface analysis of chlortetracycline and tetracycline productin with k-carrageenan immobilized *Streptomyces aureofaciens*. *J. Enz. Microb. Technol.*, 29: 314-320.

Azevedo, E.C., E.M. Rios, K. Fukushima and C.G.M. Takaki. 1993. Bacitracin production by a new strain of *Bacillus subtilis*. Extraction, purification and characterization. *Appl. Biochem. Biotechnol*., 42: 1-7.

Bottone, E.J. and R.W. Peluso. 2003. Production by *Bacillus pumilus* (MSH) of an antifungal compound that is active against *Mucoraceae* and *Aspergillus* species: preliminary report. *J. Med. Microbiol*., 52: 69-74.

Cane, D.E., C.T. Walsh and C. Khosla. 1998. Harnessing the biosynthetic code: combinations, permutations, and mutations. *Science*, 282: 63-68.

Chitnis, S.N., D.B. Kameswari and K.S. Prasad. 1993. Induction of autolysis in starved Escherichia coli cells by seminal plasmin, an antimicrobial protein from bovine seminal plasma. *FEMS Microbiol. Lett.*, 112: 147-150.

Egorov, N.S., I. Kozlova and I.P. Baranova. 1986. Nisin biosynthesis during the joint cultivation of *Streptococcus lactis* strain MGU and yeasts. *Antibiot. Med. Biotekhnol.*, 31: 828-830.

Espeso, E.A., J. Tilburn, H.N. Arts and M.A. Peñalva. 1993. pH regulation is a major determinant in expression of a fungal penicillin biosynthetic gene. *EMBO J*., 12: 3947-3956.

Gautam, P., A. Fatu, A. Panday, G. Szakasc and C.R. Soccol. 2002. Microbial production of extracellular phytase using polystyrene as inert solid support. *Bioresour. Technol*., 83: 229-233.

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

Haavik, H.I. and O. Froyshov. 1975. Function of peptide antibiotics in producer organisms. *Nature*, 254: 79-82.

Hamedi, J., F. Khodagholi and Hassani-Nasab. 2005. Increased erythromycin production by alginate as a medium ingredient or immobilization support in cultures of *Saccharopolyspora erythraea*. *Biotechnol. Lett*., 27: 661-664.

Holt, J.G. 1993. *Bergey's Manual of Determinative Bacteriology.* 9th Ed. Williams and Wilkins, Baltimore, MD, USA,

http://www.ias.ac.in/currsci/jul10/articles17.htm. Ramakrishna, S.V. and Prakasham, R.S., Microbial fermentations with immobilized cells Biochemical and Environmental Engineering, Indian Institute of Chemical Technology, Hyderabad, India. (website last visited on May 2008)

Iglewski, W.J. and N.B. Gerhardt. 1978. Identification of an antibiotic-producing bacterium from the human intestinal tract and characterization of its antimicrobial product. *Antimicrob. Agents Chem*., 13: 81-89.

Lebbadi, M., A. Galvez, M. Maqueda, M. Martinez-Bueno and E. Valihuia. 1994. Fungicin M4: a narrow spectrum peptide antibiotic from *Bacillus licheniformis* M-4. *J. Appl. Bacteriol.*, 77: 49-53.

Mendo, S., N.A. Faustino, A.C. Sarmento, F. Amado and A.J. Moir. 2004. Purification and characterization of a new peptide antibiotic produced by a thermotolerant *Bacillus licheniformis* strain. *Biotechnol. Lett*., 26: 115-119.

Morikawa, Y., L. Karube and S. Suzuki. 1979. Penicillin G production by immobilized whole cells of *Penicillium chrysogenum*. *Biotechnol. Bioeng*., 21: 261-270.

Park, C.B., H.S. Kim and S.C. Kim. 1998. Mechanism of action of the antimicrobial peptide buforin II: buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions. *Biochem. Biophys. Res. Commun*., 244: 253-257.

Romo, S. and C. Perezmartinez. 1997. The use of immobilization in alginate beads for long-term storage of *Pseudoanabaena-Galeata* (Cyanobacteria) in the laboratory. *J. Phycol*., 33: 1073-1076.

Schallmey, M., A. Singh and O.P. Ward. 2004. Developments in the use of *Bacillus* species for industrial production. *Can. J. Microbiol*., 50: 1-17.

Smet, K.D. and R. Contreras. 2005. Human antimicrobial peptides: defensins, cathelicidins and histatins. *Biotechnol Lett.,* 27: 1337-1347.

Solé, M., A. Francia, N. Rius and J.G. Lorén. 1997. The role of pH in the "glucose effect" on prodigiosin production by non-proliferating cells of *Serratia marcescens*. *Lett. Appl. Microbiol*., 25: 81-84.

Srinivasulu, B., K. Adinarayana and P. Ellaiah. 2003. Investigations on neomycin production with immobilized cells of *Streptomyces marinensis* NUV-5 in calcium alginate matrix. *AAPS Pharm. Sci. Tech.*, 21: 57-60.

Tonkova, A., V. Ivanova, E. Dobreva, M. Stefanova and D. Spasova. 1994. Thermostable alpha-amylase production by immobilized *Bacillus licheniformis* cells in agar gel and on acrylonitrile/acrylamide membranes. *Appl. Microbiol. Biotechnol*., 41: 517-522.

Yousaf, M. 1997. *Studies on the cultural conditions for the production of antibiotic bacitracin by B. licheniformis*. Ph.D. thesis, Islamia University, Bahawalpur.

(Received for publication 1 January 2009)