ISOLATION, IDENTIFICATION AND OPTIMIZATION OF BACITRACIN PRODUCED BY *BACILLUS* SP.

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Abstract

Bacillus subtilis and *Bacillus pumilus* were isolated from soil and screened for the production of antibiotics by plate assay and then cultured in shake flask fermentation at 30ºC for further studies. Identification of antibiotics was done by paper chromatography. Bacitracin was found to be produced by both the strains against *Micrococcus luteus* (ATCC# 10240), whereas; *Staphylococcus aureus* (ATCC# 6538) proved to be resistant to Bacitracin produced by *Bacillus pumilus.* The maximum production of Bacitracin from *B. subtilis* and *B. pumilus* against *Staphylococcus aureus* and *Micrococcus luteus* at different pH (6-9), incubation time (0-144 hours) and glucose concentration (1-5%) was checked by agar diffusion assay as detected by the size of zones of inhibition. Maximum zones of inhibition were observed at pH 8, 5% glucose and after 24 hours of incubation at 30ºC against *Staphylococcus aureus* and *Micrococcus luteus*.

Introduction

The word "antibiotic" is derived from Greek term antibiosis, which literally means "against life". It can be purified from microbial fermentation and modified chemically or enzymatically for either chemical use or for fundamental studies (Robbers *et al*., 1996; De Mondena *et al*., 1993). 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* (Zinsser *et al*., 1988). 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* (Todar, 2002; Claus & Blackwill, 1989). In pharmaceutical industry, several peptide antibiotics of importance are produced by *Bacillus* species such as bacitracin, polymyxin, gramicidin, tyrocidine, subtilin, bacilysin etc. Bacilli are rod-shaped, Gram-positive, sporulating, aerobes or facultative anaerobes. Most bacilli are saprophytes. Each bacterium creates only one spore, which is resistant to heat, cold, radiation, desiccation, and disinfectants. Bacilli exhibit an array of physiologic abilities that allow them to live in a wide range of habitats, including many extreme habitats such as desert sands, hot springs, and Arctic soils. Species in the genus *Bacillus* can be thermophilic, psychrophilic, acidophilic, alkaliphilic, halotolerant or halophilic and are capable at growing at pH values, temperatures, and salt concentrations where few other organisms can survive (http://biology.kenyon.edu/Microbial_Biorealm/bacteria/gram-positive/bacillus/bacillus.htm). Most of the peptide antibiotics produced by bacilli are active

against gram-positive bacteria; however, compounds such as polymyxin, colistin and circulin exhibit activity almost exclusively upon gram-negative bacteria, whereas bacillomycin, mycobacillin and fungistatin are effective against molds and yeasts (Katz & Demain, 1977). Berdy (1974) reported the production of 167 peptide antibiotics from *Bacillus subtilis* and *Bacillus brevis.* Of this total, 66 different peptide antibiotics are elaborated by strains of *Bacillus subtilis* and 23 are products of *Bacillus brevis*.

Materials and Methods

Isolation of bacitracin producing microorganisms: Soil samples were collected from rhizosbhere and checked for the presence of antibiotic producing microorganisms. Susceptible test organisms that is; *Micrococcus luteus* (ATCC# 10240) and *Staphylococcus aureus* (ATCC# 6538) was isolated on nutrient agar plates. Soil was sprinkled on the lawn of *M. luteus* and *S. auerus*. Plates were incubated at 30ºC for 24 hours. After incubation the plates were checked for the appearance of zones of inhibition around the colonies of microorganisms.

Identification of *Bacillus* species: Isolated strains were identified on the basis of their morphological and biochemical characteristics according to Bergey's Manual of Determinative Bacteriology (Buchanan & Gibbons, 1974).

Bacitracin production by *Bacillus* species

Inoculum preparation: Inoculum was prepared in nutrient broth by inoculating Bacitracin producing *Bacillus* species separately and incubating at 30˚C for 72 hours in an orbital shaker at 150 rpm.

Production media: About 10% inoculum of both the isolates was added in separate flasks, each containing 100 ml synthetic media for checking the Bacitracin production. Synthetic media contained (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; pH 7. Flasks were incubated at 30˚C in an orbital shaker at 150 rpm. After every 24 hours, samples were taken up to 144 hours, centrifuged to get cell free supernatants, which were sterilized through 0.2 μm filter paper.

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 *Staphylococcus aureus,* and *Micrococcus luteus* were diluted with pre-sterilized normal saline and the turbidity of the cultures was adjusted with 0.5 McFarland, bacterial lawn and then wells were prepared over the nutrient agar plates. About 80 μl cell free supernatants were added in the wells and the plates were incubated at 37ºC for 24 hours. After 24 hrs, the zones of inhibition were observed and measured.

Identification of peptide antibiotic by paper chromatography: Method developed by Snell *et al*., (1955) was used for the identification of peptide antibiotics. Ascending chromatograms on Whatman No.1 were developed containing 1 cm of solvent mixture (Acetone: Acetic acid: Water, 50:3:47). Samples were spotted 2 cm above the base of the

paper, and dried thoroughly before placing in the solvent. After the solvent had migrated to the top of the paper (usually 12 cm) the chromatograms were air dried and exposed to steam to ensure adequate removal of acetic acid. The migrated antibiotics were detected bioautographically where papers were placed for one-half hour on nutrient agar plates seeded with the *Staphylococcus aureus* and *Micrococcus luteus.* Plates were then incubated at 37°C for 17 hours. Finally the relative flow (R.f) values of the migrated antibiotics were determined and compared with the R.f values of different antibiotics as provided by Snell *et al*., (1955).

Optimum conditions for antibiotic production: Time of incubation (0-144 hrs), pH (6, 7, 8, 9) and glucose concentration (1-5%) in the production medium were optimized for maximum production of antibiotic by *B. subtilis* and *B. pumilus.* Both the strains were incubated at 30°C in an orbital shaker at 150 rpm and the samples were taken after every 24 hours.

Results

Isolation of bacitracin producing microorganisms: A total of five bacterial strains were found to be producing zones of inhibition, out of which two species were selected on the basis of maximum zone of inhibition for further studies.

Identification of bacitracin producing microorganisms: The bacteria were identified as *Bacillus subtilis* and *Bacillus pumilus* on the basis of their morphological and biochemical characteristics (Buchanan & Gibbons, 1974) (Table 1 to 2).

Table 1. Morphological tests for identification of *Bacillus subtilis* and *Bacillus pumilus*.

Test	Result	Result
Grams staining	+	+
Shape	Coccobacilli	Bacilli
Spore formation	+	+
Motility	+	+

Table 2. Biochemical tests for identification of *Bacillus subtilis* and *Bacillus pumilus*.

Test	Result	Result
Indole production	−	−
Citrate utilization	+	+
Methyl red	−	+
Voges- Proskauer	+	+
Oxidase	+	−
Catalase	+	+
Starch hydrolysis	+	−
Nitrate reduction	+	−
Casein hydrolysis	+	+
Gas production from glucose	−	−

Antibiotic production by *Bacillus subtilis* and *Bacillus pumilus*: 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 (mm) (Fig. 1).

Identification of peptide antibiotic by paper chromatography: All of the antibiotics produced by *B. subtilis* and *B. pumilus* were identified by comparing their R.f values with those provided by Snell *et al*., (1955). *B. pumilus* produced two antibiotics against *M. luteus*, one having R.f. value 1, which by comparison was found to be Bacitracin, while other having R.f. value 0.25 was not comparable with any value given by Snell *et al*., (1955) (Fig. 2).

Antibiotics produced by *B. pumilus* were not able to inhibit the growth of *S. aureus*, which showed that *S. aureus* was resistant to those antibiotics (Fig. 3). While *B. subtilis* produced only one antibiotic against *M. luteus*, having R.f value 1, which was found to be equal to that of Bacitracin (Fig. 4).

Optimum conditions for antibiotic production: Parameters like incubation period, initial pH of the medium and glucose concentration were optimized for maximum production of antibiotic by *B. subtilis* and *B. pumilus.*

Effect of incubation period: Maximum zone of inhibition was observed in case of *B. pumilus* against *S. aureus* (19mm) and *M. luteus* (17mm) after 24 hours. while after 48, 72, 96, 120 and 144 hours a gradual decrease in activity was seen against *S. aureus* and *M. luteus*. Whereas *B. subtilis* showed no activity against *S. aureus,* while against *M. luteus* maximum activity was detected (14mm), followed by a gradual decrease with time (Fig. 5 and 6).

Effect of pH: Maximum activity was detected at pH 8 against *M. luteus* by both *Bacillus* species, followed by pH 9 (Figs. 7, 8). *B. pumilus* showed best activity (18 mm) against *S. aureus* at pH 9 in 24 hours of incubation (Fig. 9), gradual decrease in activity was observed with the passage of time. No activity was detected in case of *B. subtilis* against *S. aureus.*

Effect of different glucose concentrations: *B. subtilis* showed best activity (19 mm) against *M. luteus* at 1 and 5% glucose concentrations after 24 and 96 hours of incubation respectively (Fig. 10), whereas *B. pumilus* showed best activity (26 mm) against *M. luteus* at 5% glucose concentration in 120 hours (Figs. 11, 13). *B. pumilus* showed best activity against *S. aureus* in 48 hours at all glucose concentrations (Fig. 12), however no significant activity has been detected in case of *B. subtilis* against *S. aureus*.

Discussion

There are many strains of the genus *Bacillus* which can produce a wide variety of antibiotics including bacitracin, polymyxin, colistin etc. Several bacitracins have been characterized; bacitracin A is the dominant commercial product (Schallmey *et al*., 2004). *Bacillus* antibiotics are generally produced at the early stages of sporulation. Eppelmann *et al*., (2001) 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.
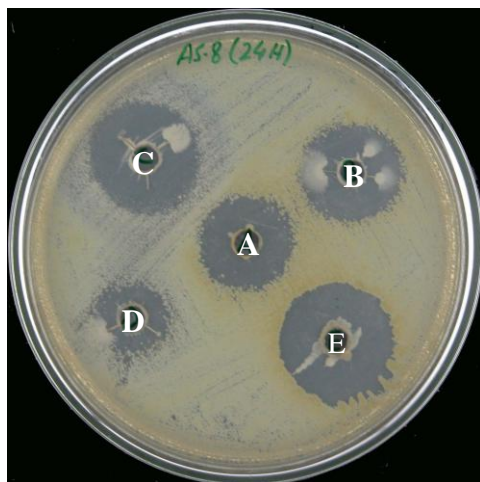
Fig. 1. *Bacillus subtilis* (C) and *Bacillus pumilus* (E) showing greater zones of inhibition against *Micrococcus luteus* at 24 hrs.
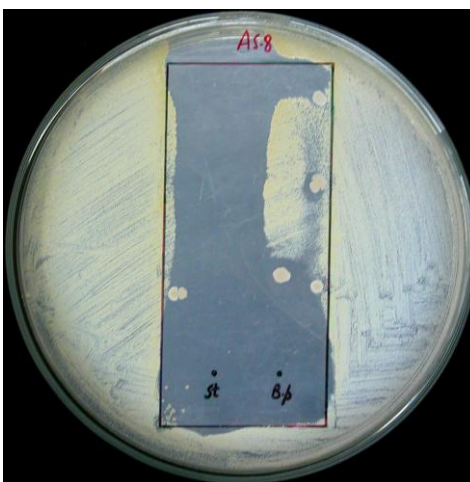
Fig. 2. Antibiotics produced by *B. pumilus* against *M. luteus.* [St: Standard Bacitracin (10mg/1ml) and B.p: *B. pumilus*]

Fig. 3. No activity was shown by *B. pumilus* against *S. aureus*; as compared with standard Bacitracin (10mg/1ml).

Fig. 4. Antibiotic produced by *B. subtilis* against *M. luteus.* [St: Standard Bacitracin (10mg/1ml) and B.s: *B. subtilis*]

The rhizobacterium *Bacillus subtilis* is an endospore forming bacteria. Several hundred wild-type *B. subtilis* strains have been collected, with the potential to produce more than two dozen antibiotics with an amazing variety of structures. *Bacillus subtilis* also produces several other antibiotics: subtilin, a 32-residue peptide; bacilysin, a dipeptide; subsporins A–C, lipooligopeptides; and rhizocticins A–D, phosphooligopeptides (Priest, 1992; DeFuria & Claridge, 1976).

The present research work was carried out to optimize the conditions for the production of bioactive microbial metabolites by *Bacillus subtilis* and *Bacillus pumilus*. The two Bacilli were isolated from soil and identified according to Bergey's Manual of Determinative Bacteriology (Buchanan & Gibbons, 1974).
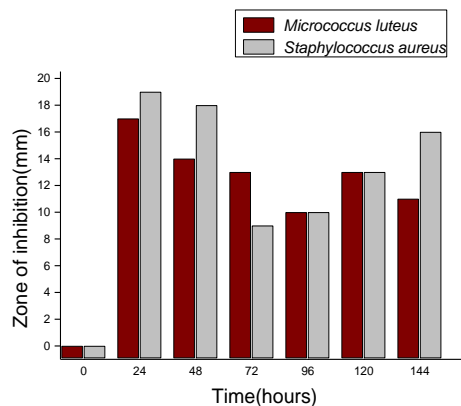
Fig. 5. Antimicrobial effect of *B. pumilus* against *S. aureus* and *M. luteus* in terms of zone of inhibition at various time intervals
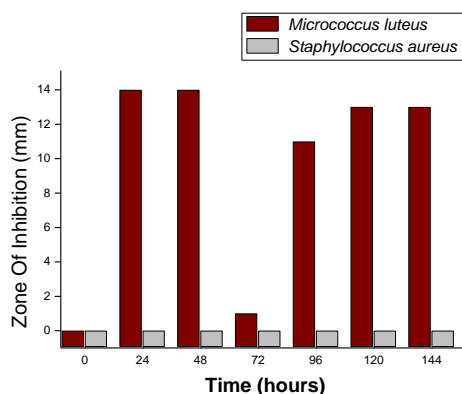

Fig. 6. Antimicrobial effect of *B. subtilis* against *S. aureus* and *M. luteus* in terms of zone of inhibition at various time intervals.
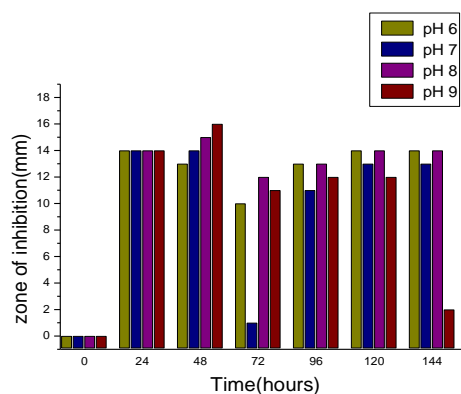

Fig. 7. Antimicrobial effect of *B. subtilis* against *M. luteus* in terms of zone of inhibition at various pH values.
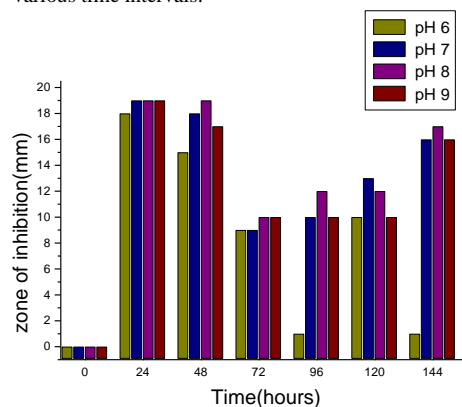

Fig. 8. Antimicrobial effect of *B. pumilus* against *M. luteus* in terms of zone of inhibition at various pH values.
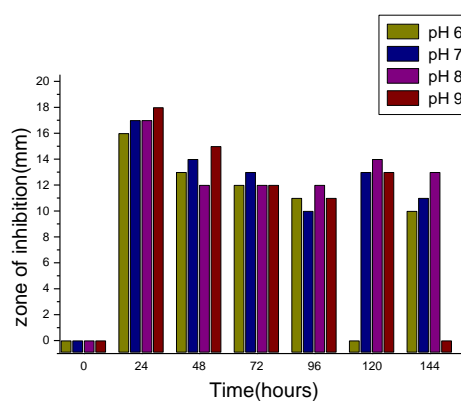

Fig. 9. Antimicrobial effect of *B. pumilus* against *S. aureus* in terms of zone of inhibition at various pH values.
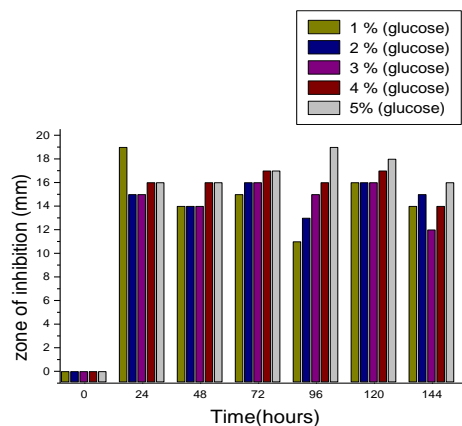

Fig. 10. Antimicrobial effect of *B. subtilis* against *M. luteus* in terms of zone of inhibition at various glucose concentrations.
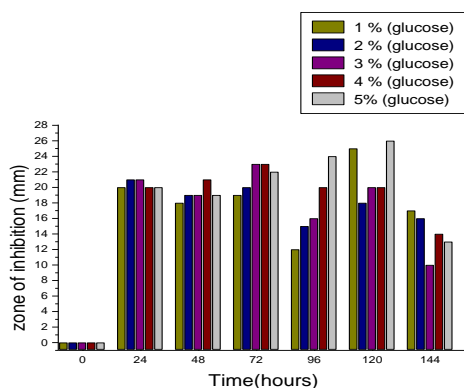
Fig. 11. Antimicrobial effect of *B. pumilus* against *M. luteus* in terms of zone of inhibition at various glucose concentrations.
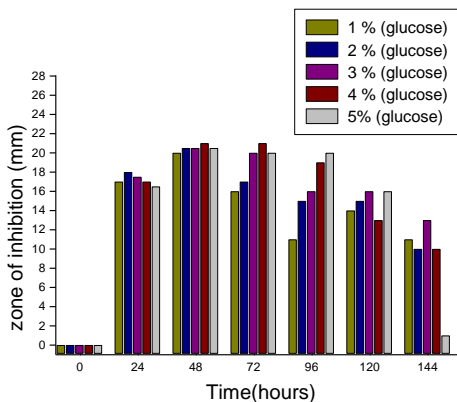
Fig. 12. Antimicrobial effect of *B. pumilus* against *S. aureus* in terms of zone of inhibition at various glucose concentrations.
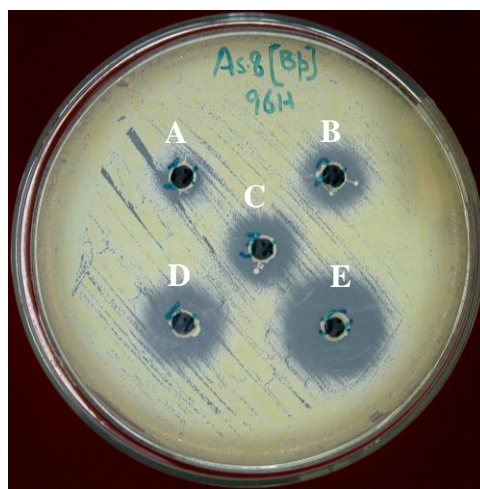

Fig. 13. Antimicrobial activity of *B. pumilus* at 96 hour against *M. luteus* at different glucose conc. as shown by zone of inhibition (mm).

In the present study the antibacterial activity of both the organisms was tested against a variety of organisms but they exhibited better activity against gram-positive *Staphylococcus aureus* and *Micrococcus luteus*. Different scientists have reported inhibition of various organisms. Marahiel *et al*., (1997) 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 *Bacillus 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 search for antibiotics produced by *Bacillus* species, especially *Bacillus cereus*, *Bacillus subtilis* and *Bacillus licheniformis*, several antifungal compounds, mainly peptides, have also been described (Katz & Demain, 1977; Kugler *et al*., 1990; Lebbadi

et al., 1994; Silo-Suh *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 both the strains at 30ºC against *S. aureus* and *M. luteus*. Therefore further studies were carried out at this temperature. Early studies by Berdy (1974) 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.

The antibiotics produced by *Bacillus subtilis* and *Bacillus pumilus* were analyzed by Paper chromatographic technique and migrated antibiotics were detected bioautographically by placing the papers on agar plates seeded with test organisms. The R.f values were compared with those given by Snell *et al*., (1955). It was found that two antibiotics were produced by *B. pumilus* against *M. luteus*. One was found to be similar to bacitracin and another unidentified compound with antimicrobial activity against *M. luteus* was also produced. *B. subtilis* produced an antibiotic with R.f value similar to that of Bacitracin against *M. luteus*.

Multiplicity of antibiotic production obviously complicates attempts to identify antibiotics in unfractionated material. However, even in whole cultures similarities or differences between known and unidentified antibiotics may be noted by means of chromatography (Snell et al., 1955). Besides Paper Chromatography there are many other methods, which can be further used for analysis of antibiotics like HPLC, FTIR etc.

In the present study production of antibiotics was studied at different time periods i.e., 0, 24, 48, 72, 96,120 and 144 hours of incubation. It was found that maximum zone of inhibition (14 mm) was produced by *Bacillus subtilis* (cell free extract) against *Micrococcus luteus* whereas, *B. pumilus* (cell free extract) showed increased inhibition (19 mm) at 48 hours against *M. luteus*. *B. subtilis* (cell free extract) have not shown any inhibition of *S. aureus* but *B. pumilus* (cell free extract) showed zone of 17 mm against *S. aureus*.

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* 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. In submerged fermentation, 20 hours old vegetative inoculum gave the maximum yield of bacitracin by *B. licheniformis* (Yousaf, 1997).

Changes in external pH affect many cellular processes such as the regulation of the biosynthesis of secondary metabolites (Chang *et al*., 1991; Datta & Kothary, 1993; Solé *et al*., 1994; Sole *et al*., 1997). Effect of pH was studied by adjusting the initial pH (6, 7, 8, 9) of the production medium. It is evident from the results that *Bacillus subtilis* (cell free extract) showed increased inhibition (16 mm) after 48 hours of incubation at pH 9, when tested against *M. luteus* whereas, *B. pumilus* (cell free extract) showed maximum zone of inhibition (19 mm) after 24 hours of incubation at pH 7, 8 and 9, and also showed similar inhibition after 48 hours at pH 8 against *M. luteus*. It has earlier been reported by Berdy (1974) that pH of 7.8-8 gave maximum production of bacitracin. Iglewski & Gerhardt (1978) have 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.

The effect of glucose concentration on the production of antibiotic was studied. Maximum zone of inhibition (26 mm) was produced by *B. pumilus* against *M. luteus* at 120 hours in 5% glucose while *B. subtilis* produced a maximum zone of inhibition (19 mm) in 1% glucose at 24 hours and in 5% glucose at 96 hours. *B. subtilis* showed no activity against *S. aureus,* and *B. pumilus* produced a maximum zone (21 mm) against *S. aureus* in 4% glucose at 48 and 72 hours respectively. 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.

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