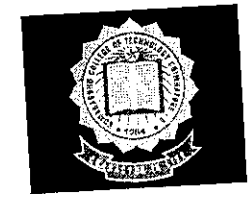

PRODUCTION OF BACITRACIN FROM *Bacillus subtilis* AND EVALUATION OF ITS ANTIMICROBIAL ACTIVITY AGAINST *E.coli*

A PROJECT REPORT

Submitted by

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In partial fulfillment for the award of the degree

of

BACHELOR OF TECHNOLOGY

IN

INDUSTRIAL BIOTECHNOLOGY

KUMARAGURU COLLEGE OF TECHNOLOGY, COIMBATORE-6

ANNA UNIVERSITY: CHENNAI 600 025

BONAFIDE CERTIFICATE

Certified that this project report **"PRODUCTION OF BACITRACIN FROM *Bacillus subtilis* AND EVALUATION OF ITS ANTIMICROBIAL ACTIVITY AGAINST *E.coli*"** is the bonafide work of **"ARUN M AND SIVARANJANI I"** who carried out the project work under my supervision.

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Lecturer & Coordinator,
Departmetn of Biotechnology

CERTIFICATE

This is to certify that Mr. M.ARUN of final year B.Tech, Industrial Biotechnology of KUMARAGURU COLLEGE OF TECHNOLOGY, Coimbatore has undergone project work titled "PRODUCTION OF A BACITRACIN FROM *Bacillus subtilis* AND EVALUATION OF ITS ANTIMICROBIAL ACTIVITY AGAINST *E.coli*" under my guidance from January 22 to April 2 , 2007.

During the project work his performance and conduct were found to be good.

Signature

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Signature

CERTIFICATE OF EVALUATION

College : KUMARAGURU COLLEGE OF TECHNOLOGY

Branch : INDUSTRIAL BIOTECHNOLOGY

Semester : Eighth Semester

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The report of the project work submitted by the above students in partial fulfillment for the award of Bachelor of Technology degree in Industrial Biotechnology of Anna University was confirmed to be the report of the work done by the above students and then evaluated.

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The present study involves the production of the antibiotic bacitracin from *Bacillus subtilis* through shake flask and bioreactor methods. A pure culture of *B.subtilis* was obtained from IMTECH, Chandigarh and checked for its genus specificity through various biochemical tests like Gram staining, catalase test, citrate test, triple sugar iron test, etc. The 24 hours old culture of *B.subtilis* was inoculated in both half strength and full strength medium for the production of bacitracin using shake flask method at 160 rpm and bioreactor method at 250 rpm. This production cycle was run for a period of 10 days and the produced bacitracin was identified using thin layer chromatography against a standard bacitracin and it was quantified using agar ditch diffusion method. Maximum antibiotic concentration was obtained on the 7^{th} day of the production cycles. It was found that half strength medium of bioreactor method produced an antibiotic concentration (39µg/20µl) which was better than half strength medium of shake flask method (31.2µg/20µl). It was also found that full strength medium of the bioreactor produced more bacitracin (52µg/20µl) than the shake flask method (44.2µg/20µl). Evaluation of antimicrobial activity of bacitracin against *E.coli* was proved to be potent in its action. The zone of inhibition against *E.coli* was found to be maximum for full strength medium of bioreactor (20mm). It is evident from the study that the bacitracin thus produced by following different methods comparatively indicated bioreactor

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The use of natural products with therapeutic properties is as ancient as human civilization and, for a long time, plant and microbial products were the main sources of drugs. The reasons for this were that pure compounds were easily obtained and structural modifications of such compounds to produce potentially more active and safer drugs could be easily achieved (Marjorie., 1999). The discovery and use of certain chemical substances termed as antibiotics which has been produced by several microorganisms through secondary metabolic pathways has been one of the major scientific achievements in the earliest of 20th century (Schallmey *et al.*, 2004) and these compounds can fight against various diseases. Generally, an antibiotic is a chemical substance, possessing a molecular weight lesser than 2 Kda and used to kill or to prevent the growth of any type of microorganisms at a lower dosage (Chopra *et al*., 1996). During the early period of antibiotic usage, the bacterial infections were considered to be tamed (Baquero and Seibert., 1997). However, certain bacterial diseases, like syphilis and cholera, were considered on their way to eradication (Macay *et al.*, 2006) through proper therapeutic measures and in this aspect the antibiotics play a vital role.

Antibiotics can be classified on two bases.

1. Based on the ability to kill the microorganisms

2. Based on their structure

c) Aminoglycosides
d) Cephalosporins
e) Macrolides
f) Polypeptides
g) Penicillins
h) Tetracyclines

TABLE 1.1. ANTIBIOTICS AND ITS USES

ANTIBIOTIC	USES
Aminoglycosides	Used to treat infections caused by Gram-negative bacteria, such as *E.coli* and *Klebsiella* and *P.aeruginosa*
Cephalosporins	To treat gastrointestinal upset, diarrhea and nausea.
Macrolides	To treat nausea, vomiting and diarrhea.
Polypeptides	For the treatment of eye, ear and urinary bladder infections.

The polypeptide antibiotic bacitracin produced by various strains of *Bacillus licheniformis* exhibits a pronounced antimicrobial activity primarily against Gram negative bacteria and a few Gram positive bacteria. It is one of the important antibiotic used in human medicine and also used in animal husbandary for the prevention and control of diseases existing in farm animals. Generally, bacitracin is a mixture of several polypeptides differing in their amino acid composition (Hammes and Frank., 1979).

Since, the world population is largely dependent on antibiotics; we considered it is worthwhile to work on antibiotics. Also since, production of bacitracin from *Bacillus subtilis* is not established thoroughly, we took this topic as our project with the following objectives.

1. To confirm the genus specificity of commercially obtained *B.subtilis* (ATCC 6633) using various biochemical tests.
2. To produce bacitracin in half strength and full strength media from *B.subtilis* through shake flask and bioreactor methods.
3. To detect the presence of bacitracin by Thin layer chromatography
4. To quantify the produced bacitracin by agar ditch diffusion method.
5. To evaluate the antimicrobial activity of bacitracin against commercially obtained *E.coli* (ATCC 10536).

Bacillus subtilis is a rod shaped, single celled, gram positive, motile micro organism with peritrichous flagellar arrangements. Their size ranges from 0.5-1.0μm wide and 1-4μm long. They are capable of producing proteolytic and hydrolytic enzymes to degrade the complex nutrients into simpler forms. They have the ability to produce resistant survival forms termed as endospores. These endospores are resistant to heat, drying, radiation and various chemical disinfectants. They have another survival weapon that is they are capable of producing peptide antibiotics such as subtilosin, diffidicin, bacitracin, bacteriocin, etc (Pfeffer *et al.*, 1991).

Peptide antibiotics form a unique group of bioactive molecules (Schroder., 1999). Many peptide antibiotics have novel structural motifs, such cyclic structures, contain uncommon amino acids especially D-form amino acids and are often further modified and conjugated with sugars, lipids and other molecules. Depending on their amino acid components and their conjugates, the mechanism of action may vary dramatically (McCafferty and Weinberg., 1999). Examples of peptide antibiotics include some well known or commonly used drugs (Aversa and Rojers., 1997) such as polymixin, colistin, dactinomycin, teichoplanin, vancomycin, virginiamycin.

Bacitracin was first discovered in 1943 and named after a culture of *Bacillus* and the last name of a seven year old American girl, Margaret Tracey, from whose

(Venkateswerlu., 1981). It is an ingredient in several commercially available topical triple antibiotic ointment such polysporin and neosporin that are used to prevent infection is minor cuts and burns (Arky., 1997). Although this antibiotic has been generally considered safe for topical use, it has recently been found in a few cases to generate delayed hypersensitivity, acute IgE mediated allergic reactions and even life threatening anaphylaxis (Dyck and Samson., 1997). Bacitracin is not administered systematically as it is nephrotoxic and is used only as a last option. It is considered safe when taken orally as the gastrointestinal tract does not absorb significant amount of drug (Arky., 1997). Bacitracin has thus been used for the treatment for gastrointestinal infection and was found to be as effective as vancomycin (Dudley and Matsuhasi., 1986) against vancomycin-resistant *Enterococcus faecium* (O'Donovan *et al.*, 1994) and the intestinal infections by *Entamoeba histolytica* (Andrew., 1995). It has been widely used as an animal feed additive to improve animal body weight and prevent diseases in farm animals (Abdulrahim and Rafiq., 1999). Consequently, Bacitracin is important in both the pharmaceutical and livestock industries and is produced in large quantities through out the world.

Bacterial resistance against antibiotics has become a threatening health issue in recent years. However, bacterial resistance of bacitracin is still scarce despite its wide use in the past several decades. Thus, it can serve as a potential lead for

2.1. BACTERIAL CONGENERS

Bacitracin is produced as a mixture of closely related congeners by *Bacillus subtilis* or *Bacillus licheniformis* (Ziffer., 2002). Soon after its discovery the mixture of crude bacitracin was separated into several components by the use of counter current distribution (CCD technique) (Newton *et al*, 1953). In the 1970s, HPLC began to replace the old CCD technique for bacitracin purification (Epperson and Ming., 2000). Eventually, over 30 different minor components were separated from the crude mixture and analyzed by the use of fast atom bombardment, tandem mass spectrometry and electron spray ionization, mass spectrometry to establish their structures (Morris., 1994). A recent capillary chromatographic study further isolated bacitracin into more than 50 peaks. Several different nomenclatures have been used to classify the bacitracin congeners.

Bacitracins B_1, B_2, B_3 have the same sequence as bacitracin A except that Ile^1, Ile^5 and Ile^8 respectively are substituted by valine. Similarly the substitutions of Val for Ile at the positions 1, 5 and 8 with different combinations affords bacitracins D and E. in addition to these congeners, a few modified bacitracin derivatives have also been prepared chemically or isolated from the crude mixture (Stachelhaus and Maraheil., 1996). For example, the biologically inactive bacitracin F can be obtained by air oxidation of bacitracin A_1 in a slightly alkaline aqueous solution, in which the amino methylene-thiazoline moiety is converted

amounts. It is also known that bacitracins A and B accounts for approximately 95% of the production of biological activity of the crude bacitracin mixture.

2.2. BIOSYNTHESIS OF BACITRACIN:

The biosynthesis of many peptides and polyketides and their hybrid conjugates follows a non-ribosomal pathway catalyzed by large clusters of peptide and ketide synthetases and peptide/ketide hybrid synthetases respectively (Moffitt and Neilan., 1965).

Like those structurally diverse peptides and polyketones, bacitracin congeners are also non-ribosomal products of a large peptide synthetase complex (Ogawa *et al.*, 1981). The structure and mechanism of bacitracin synthetase resembles those of other peptide and polyketide synthetases, which are comprised of a multi domain modular structure for the catalysis of initiation of the synthesis via ATP- activated formation of thioester linkage to the enzyme, elongation mediated by condensation of the thioester-linked aminoacid and\or peptide on the peptide carrier domain followed by a mechanism not yet fully understood and termination of the peptide or polyketide chain by a thioesterase domain via transfer of the final product to a serine in the thioesterase followed by hydrolysis (Eppelmann*et al.*, 2001). The reactant aminoacids or carboxylates are specifically

The branched of cyclic dodecypeptide antibiotic bacitracin, produced by special strains of Bacillus is synthesized nonribosomally by a large multienzyme complex composed of three bacitracin synthetases BA1, BA2 and BA3. These enzymes activate and incorporate the constituent amino acids of bacitracin by a thiotemplate mechanism in a pathway driven by a protein template. The biochemical features of these enzymes have been studied intensively but little is known about the molecular organization of their genes

The entire bacitracin synthetase operon containing the genes bacA-bacC was cloned and sequenced, identifying a modular structure typical of peptide synthetases. The bac-A gene product (BA1, 598kDa) contains five modules, with an internal epimerization domain attached to the fourth; bacB encodes BA2 (297 kDa), and has two modules and a carboxy-terminals epimerization domains; back encodes BA3, five modules (723 kDa) with additional internal epimerization domains attached to the second and fourth. A carboxy-terminal putative thioesterase domain was detected in BA3. A putative cyclization domain was found in BA1 that may be involved in thiazoline ring formation. The adenlytion/thioester binding domains of the first two BA1 modules were over produced and the detected amino-acid specificity coincides with the first two amino-acid in bacitracin. Disruption of chromosomal bacB resulted in a bacitracin deficient mutant (Derefahrt *et al.*, 2004)

fragment of *B. licheniformis*) and expression of these genes in *E.coli*. Indicating that the genes for bacitracin synthesis are clustered in the genome of *B.licheniformis*. This proposition was supported by isolation of a Tn917-generated bacitracin deficient mutant with an insertion in the vicinity of the bacB gene, but to date the sequences of the genes have not been available (Pfeffer *et al.*, 1991)

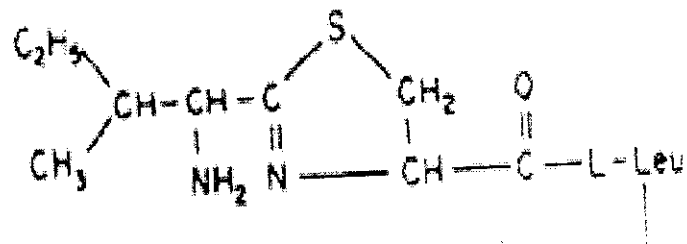
Analysis of the sub cloned nucleotide sequence revealed three huge open reading frames (ORFs) with the same direction of transcription. The first ORF, designated bacA, is 15768 bp in length and codes for a protein of 5255 amino acids and a predicted mass of 598,379 Da. The identified ORF, of 7824 bp, is named bacB, and codes for a protein of 2607 amino acids with a calculated mass of 297428 Da. The derived amino acid sequence is composed of two AT domains, each coupled with an amino terminal C domain. The ORF3, designated bacC, spans 6359 amino acids with a predicted mass of 722,943 Da. Taken together the three genes bacA, bacB, bacC span a stretch of 42,886 bp. There are several putative star codons for bacB; but the ATG at bp 16191, which is located 112 bp downstream of the bacA stop codon, seems to be the most probable. The bacB and bacC genes are separated by 105bp (Duerfahrt *et al.*, 2004).

Cyclization (Cy) domains in NRPS catalyze the hetero- cyclization of cysteine and serine/threonine to thiazoline and oxazoline rings. A model system consisting of the first two modules of bacitracin synthetase A fused to the

peptide bond formation and heterocyclization, several residues of the BacA1-2-Te Cy domain were analyzed by mutagenesis. Two mutants exhibited formation of the noncyclic dipeptide, providing clear evidence for the independence of condensation and cyclization (Duerfahrt *et al.*, 2004)

2.3. STRUCTURE OF BACITRACIN:

The bacitracins have certain special structural features. Firstly there are four right-handed D-amino acids alternating with the usual L-amino acids along the peptide chain. Secondly, the several charged side chains enhance the possibility of interaction with other proteins. Thirdly, there is a thiazoline ring close to the N-terminus. The thiazoline ring is though to be synthesized by a cyclic condensation of the isoleucylcysteine moiety during the process of bacitracin biosynthesis. Fourthly, the side chain of L-Lys-6 forms a link to the chain of L-Asn-12, producing a cyclic structure (Pfeffer *et al.*, 1991).



The first accepted structure for bacitracin A1 was proposed in mid-1960s (Ressler., 1966). Bacitracin A1 has been determined to be a cyclic dodecapeptide by means of chemical, spectroscopic and crystallographic techniques. It contains an unusual thiazoline ring formed by condensation of the Ile^1 carboxyl group with the –SH and –NH groups of Cys^2, a cyclic heptapeptide structure formed via an amide linkage between the side chains of $-NH_2$ of Lys^6 and the C-terminus Asn^{12} and four D-aminoacids including D-Glu^4, D-Orn^7, D-Phe^9 and D-Asp^{11}. The unusual structural features may protect this peptide from degradation by proteases (Chalker., 2000). Bacitracin A was chemically synthesized in the year 1996 which confirmed the structure of this antibiotic peptide produced by microorganism.

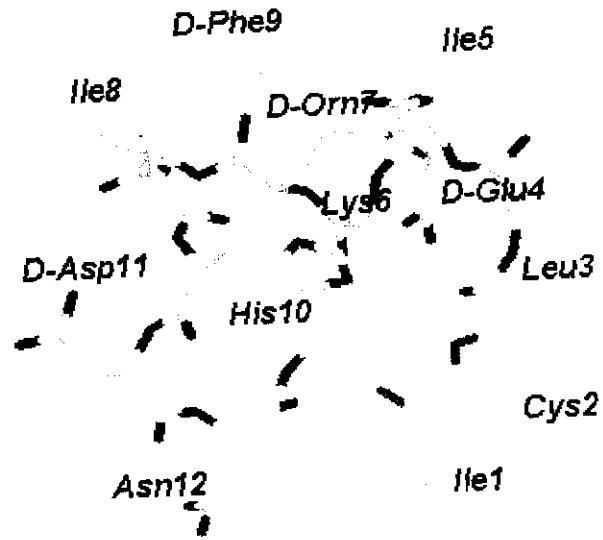


FIGURE2.2. 3-D STRUCTURE OF BACITRACIN

and His^{10} in close proximity to form a potential metal binding site in solution. The aminoacid side chain of Phe^{9} and Ile^{8} are close to Leu^{3} on the basis of nuclear overhauser effect (NOE) measurements.

The single imidazole nucleus of L-histidine residue in bacitracin-A seems to be important for the anti-bacterial activity of the molecule, since iodination, carboxymethylation and coupling of diazobenzene sulfonic acid to the histidine residue in the antibiotic caused 90-94% loss of antibacterial activity of the antibiotic. In contrast, the bacitracin sulfone and sulfoxide derivatives are active as the parent antibiotic.

2.4. METAL BINDING

Bacitracin has been known to bind several divalent metal ions to form 1:1 complexes (Selzer and Park., 1956). An order for the metal binding affinity of divalent metal ions has been established as $Cu^{2+} > Ni^{2+} > Co^{2+} \sim Zn^{2+} > Mn^{2+}$ (Ljubisa *et al.*, 1977). The biological activity of this peptide antibiotic has also been determined to be associated with divalent metal ions (Adler and Granger., 1962). Bacitracin was first suggested to bind to metal via its His^{10} imidazole and the thiazoline ring, but not the carboxyl groups. The thiazoline ring, nitrogen or

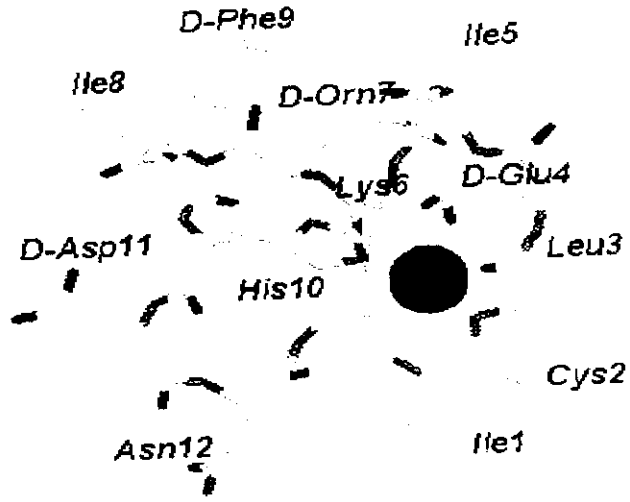


FIGURE 2.3. 3-D STRUCTURE OF BACITRACIN WITH A DIVALENT METAL ION

2.5. MECHANISM OF ACTION

Various biochemical lesions induced by bacitracin have been reported. Several effects, such as inhibition of induced enzyme synthesis and stimulation of efflux of K^+ ions, could be ascribed to the alterations in the cell membrane functions. The antibiotic also inhibits the incorporation of aminoacids into cell walls and induces the accumulation of uridine-nucleotide precursors of the wall under conditions in which incorporation of aminoacids into protein is unaffected. However, since bacitracin affects protoplasts of bacteria, its action is not limited to

intermediates, inhibition of aminoacid incorporation into the cell wall and cell lysis (Smith *et al*, 1994).

The peptidoglycan layer is constructed from repeating disaccharide units of N-acetyl glucosamine (GlcNAc) and peptidyl N-acetylmuramic acid (MurNAc) connected by β-1, 4-glycosidic bonds (Bhagavan., 2001). The peptide includes L-Ala, D-Glu, L-Lys, and one or two D-Ala are attached to MurNAc via an O-lactyl group at the carbon-3-hydroxyl position. During the peptidoglycan synthesis, the UDP-MurNAc-pentapeptide conjugate is attached to the luminyl side of endoplasmic reticulum membrane via undecarenyl monophosphate. Addition of GlcNAc via a β-1, 4-glycosidic bond forms a disaccharide complex, β-1, 4-GlcNAc-(3-pentapeptidyl-MurNAc)-O-PO$^-$-O-PO2-O-undecaprenyl. At last the disaccharide is released from the long chain undecaprenol and incorporated into the peptidoglycan layer by a β-1, 4-glycosidic bond. Cross-strand linking between the side chains –NH2 of Lys^3 with $D\text{-}Ala^4$ (while $D\text{-}Ala^5$ is lost during the cross link) also occurs at this stage and imparts strength and rigidity to the cell wall. The peptidyl sugar-carrying molecule is released at the last stage as undecaprenyl pyrophosphate, which is dephosphorylated to regenerate its monophosphate form by a membrane bound pyrophosphatase. The monophosphate form then binds to another UDP-MurNAc-pentapeptide to begin a new cycle of peptidoglycan biosynthesis. This biosynthetic pathway is analogous to that of glycoproteins in eukaryotes wherein the undecaprenyl analogue, dolichol phosphate instead serves

suggests that metallobacitracin may interfere with cell wall synthesis by tight binding to undecaprenyl pyrophosphate, which prevents its hydrolysis as a result, undecaprenyl monophosphate becomes much less available for the binding to the UDP- MurNAc-pentapeptide complex to initiate the second stage of the peptidoglycan biosynthesis. Hence the biosynthesis of the bacterial cell wall is inhibited. This mechanism has also been suggested to inhibit the synthesis of the peptidoglycan-like psuedomurein in the Methanobacterium spp. (Hammes and Frank., 1979).

3.1. IDENTIFICATION OF *Bacillus subtilis* USING BIOCHEMICAL TESTS

3.1.A. SAMPLE COLLECTION

A culture of *Bacillus subtilis* (ATCC 6633) was obtained from IMTECH laboratories, Chandigarh, India and was checked for the purity of the strain using a series of biochemical tests.

3.1.1. GRAM STAINING METHOD

Grams' staining was performed to differentiate the gram positive with gram-negative bacteria. *Bacillus subtilis* is a gram positive bacterium. In order to confirm whether the culture given was gram positive or not we did this biochemical test. A drop of cell suspension was taken in a microscopic slide and heat fixed. One drop of crystal violet was added and waited for one minute for proper staining. The contents were then washed with slow running tap water. The primary stain crystal violet stained all the cells purple by binding to the cell wall of the bacteria. A drop of Grams iodine was added and waited for 1 min. Grams iodine being a mordant formed an insoluble complex by binding to component of

purple. The counter staining was done by the addition of saffronin and waited for about 45 seconds. Saffronin stained only gram-negative cells to red since these cells had been decolorized by alcohol due to its high lipid concentration in the outer membrane. Finally, the slide was washed with tap water and dried and then examined under microscope.

3.1.2. HANGING DROP METHOD

Many bacteria are capable of motility (the ability to move under their own power). Most motile bacteria propel themselves by special organelles termed flagella. *Bacillus subtilis* is motile organism therefore this test was performed to confirm whether the culture given was motile or not. The bacterial flagellum is non-contractile, semi-rigid, helical tube composed of protein and anchors to the bacterial cytoplasmic membrane and cell wall by means of disk like structure. The rotation of the inner disk causes the flagellum to act much like a propeller. So, the bacterial motility constitutes unicellular behavior. In other words, motile bacteria are capable of a behavior called taxis. Taxis are a motile response to an environment stimulus and function to keep bacteria in an optimum environment.

The test was carried out by taking a clean glass slide with Vaseline applied

3.1.3. STARCH HYDROLYSIS

Starch is a high molecular weight polysaccharide was composed of glucose molecules and has been linked with α-(1-4) and branched with α-(1-6) glycosidic bonds. α-amylases produced by *Bacillus* species can hydrolyse the starch molecules into maltose and then to glucose. This can be tested for *Bacillus* species by using iodine. Formation of blue color indicated the presence of starch in the medium and hence the absence of amylase and *Bacillus* species. The clear zone formation indicated the absence of starch and therefore the presence of *Bacillus* species

100ml of starch agar was prepared and sterilized at 121°C for 15 minutes. The agar was poured into the petridish and allowed to solidify. The test organism was then cultured over starch agar and incubated for 48 hrs.

3.1.4. CATALASE TEST

Catalase is the name of an enzyme found in most bacteria, which initiates the breakdown of hydrogen peroxide (H_2O_2) into water (H_2O) and free oxygen

ions and combines them with half a molecule of oxygen (an oxygen atom) to form water (H_2O). During the process, energy is given off and is trapped and stored as ATP. Water is then a harmless end product. Some cytochromes in the electron transport system, however, form toxic hydrogen peroxide (H_2O_2) instead of water and this must be removed. This is done by the enzyme catalase breaking the hydrogen peroxide into water and oxygen as shown above. This test was done to prove whether the culture that was given produced the enzyme catalase or not. Trypticase soy agar was considered optimum for being used in this test.

100ml Trypticase soy agar was prepared in distilled water and sterilized at 121°C for 15 minutes. Agar was poured into the Petri dish and allowed to solidify. The test organism was then cultured over soy agar and incubated at 37°C for 48 hrs. After incubation, 2-3ml of the substrate H_2O_2 was added over the medium.

3.1.5. CITRATE TEST

The ability of the organism to utilize citrate as a sole of carbon is very useful in differentiation characteristics. Citrate utilization test determines the capability of some organisms to utilize citrate as a sole source of carbon. Here, sodium citrate was the source of carbon. Utilization of citrate can be observed by the color change

Simmon citrate agar was prepared in distilled water and sterilized at 121°C for 15 minutes. Sterilized simmon citrate agar was poured into the Petri dish and allowed to solidify. The organism was then cultured over the agar and incubated at 37°C for 48 hrs.

3.1.6. CASEIN HYDROLYSIS

Casein is the predominant protein in milk. Its presence causes milk to have its characteristic white color. Many bacteria produce caseinase or protease that cleave the protein into more soluble transparent protein derivatives. Protein hydrolysis is referred as proteolysis or peptonization. So, if proteolysis occurs, a zone of clearance is observed around the bacterial growth area. Bacillus species produce caseinase. In order to confirm our culture also produced caseinase we did this biochemical test.

100 ml of skim milk agar (obtained from HIMEDIA) was prepared using distilled water and was sterilized at 121°C for 15 minutes. After sterilization the media was poured onto the Petri plate and allowed to solidify. After solidification the plate was inoculated with *Bacillus subtilis* and kept in an incubator at 37°C for 48 hours.

incorporated into other cellular aminoacids or perhaps into the coenzyme. *Bacillus* species are not known to have the capability of breaking down sulfur containing compounds. To test H_2S production, a medium with sulfur containing components and iron is used. If sulfur is reduced H_2S will be produced, it will combine with iron salt to form a visible black iron sulfide in the tube.

100 ml of Triple sugar iron agar was prepared in distilled water and sterilized at 121°C for 15 minutes. Sterilized agar medium was poured into the Petri dish and allowed to solidify. To the solidified triple sugar iron agar slant, the microorganism was inoculated by stabbing and incubated at 37°C for 48 hrs.

3.1.2. INOCULATION OF *Bacillus subtilis*

The pure strain of *Bacillus subtilis* from various biochemical tests was used for the production of bacitracin. The culture was inoculated in the nutrient broth medium and incubated for its mass multiplication. This pure culture was used as the mother culture.

Appropriate amount of nutrient broth was weighed and dissolved in distilled

3.2. PRODUCTION OF BACITRACIN FROM *Bacillus subtilis*

3.2.1. SHAKE FLASK CULTURE METHOD FOR THE PRODUCTION OF BACITRACIN

A full strength and a half strength synthetic fermentation medium (Appendix 1) were prepared and the pH was adjusted to 6.8 to 7.0 with sodium hydroxide. The medium was then distributed in 100ml quantities into 250ml conical flasks and autoclaved at 15 lb steam pressure for 20 minutes. The sterilized medium is allowed to cool for bearable warmth. Sufficient concentrated glucose solution was then added aseptically to each bottle to give a concentration of 1%. The optimum temperature for the fermentation to take place was 37^0C. One loop ful of 24 hours culture of *Bacillus subtilis* was inoculated in both the mediums. The flasks were then placed in a orbital shaker at 160rpm at 37°C for 10 days.

EXTRACTION

1 ml of the medium was taken everyday from the both the flask aseptically. It was filtered for clarification using Whattman filter paper. The clarified medium was extracted twice with n- butanol and ether (50% n-butanol and 50% ether) in a separating funnel using half the volume of the solvent for each extraction. Concentrated hydrochloric acid was added in drops along the sides of the wall until it separates into two layers. Bacitracin was found in the lower layer. The filtrate

3.2.2 STIRRED TANK METHOD FOR THE PRODUCTION OF BACITRACIN

The working volume of stirred tank reactor was 2 liters. The airflow rate was 20lit/hr and run at 250rpm. The pH is maintained at 7.0. The optimum temperature of 37°C was maintained.

The full strength medium was prepared and sterilized inside the fermentor by passing hot air around the jacket at 121°C for 15 minutes. After cooling the media, cells of *B.subtilis* was added to the media (10% of the media). The fermentor was run for 10 days and the same procedure was followed for the half strength medium. Samples were collected everyday and the same extraction procedure as of shake flask method is carried out to calculate the antibiotic concentration.

3.3. DETECTION OF BACITRACIN

The bacitracin can be detected through thin layer chromatography. TLC works on the principle that it separates the components based on the molecular size and shape on the adsorbent. The separation occurs due to the capillary movement

temperature for 15-30mins. They were heated in an oven to activate the adsorbent. The sample (crude bacitracin) was applied on one side of the adsorbent. It was allowed to dry so that spotting can be done repeatedly for a more concentrated sample spot. The standard bacitracin was applied on the other side of the plate and allowed to dry for repeated spotting. The developing solvent was prepared with n-butanol: acetic acid: water in the ratio of 4: 1: 2, and was poured into the tank to a depth of 1cm. The TLC plate was placed vertically into the tank with the spotted end dipped in the solvent. Separation of the compound occured as the solvent moved upwards. Once the solvent reached the top of the plate, it was removed from the tank and proceeded for further identification.

3.4. QUANTIFICATION OF BACITRACIN

The activity of the antibiotic Bacitracin present in the fermented material was determined by agar diffusion method. One unit of Bacitracin activity is the amount of antibiotic in 0.2 ml of culture supernatant broth that will cause a 1mm inhibition zone outside the cylinder (Bernlohr and Novelli., 1960). One unit of Bacitracin is equivalent to 26 µg of USP standard (Harvey., 1980).

holes 8 cm of diameter were made in the plates aseptically with stainless steel borer of uniform edge and size.

Standard solution of Bacitracin was prepared by dissolving 65.2 mg of Zn Bacitracin in 1N HCl. Two opposite holes were filled with working standard of 1:4 dilution and the remaining two were filled with sample to be determined of 1:4 dilution using micropipette. 0.2 ml solution was poured in each digged hole. The plates were then placed in incubator for 24 hours at 37°C. Clear zones of inhibition were developed both by standards and samples. Diameters of zones of inhibition were measured.

3.5. ASSESSMENT OF ANTIBACTERIAL ACTIVITIES BY DISC-DIFFUSION METHOD (KIRBY-BAUER).

The spread plate technique requires a previously diluted mixture of micro-organism. During inoculation, the cells are spread over surface of the solid agar medium with a sterile L-shaped bent rod. The cells are pushed into separate areas on the surface so that they can form individual colonies.

The test organisms were selected on the basis that they cause a lot of infections in humans. The microorganism were used for the study is commercially obtained strain *Escherichia coli* (ATCC 10536).

aseptically with the test microorganism. The inoculated broth was incubated at 37°C for 24 hour. The procedure was repeated for each bacterial species.

0.2mL of the bacterial suspension was pipetted into the appropriately labeled petri dish and spread uniformly on the Nutrient agar surface. 5mm diameter paper discs were dipped in the plant extracts for 3 minute and placed on the agar surface. The plates were incubated at 37°C for 24 hours after which zones of inhibition was taken to be the diameter of the zone visibly showing the absence of growth including the diameter of the paper disc. The zones of inhibition were measured and results were recorded.

4.1. BIOCHEMICAL TESTS:

The biochemical tests were carried out to confirm whether the culture obtained from IMTECH, Chandigarh was *Bacillus subtilis*. The genus specificity was confirmed by the carrying out following tests.

GRAM STAINING

Gram staining was performed to confirm whether the organism was gram positive or gram negative. The cells were stained purple and they were rod shaped when they were viewed under the microscope. Therefore it was confirmed that the micro organism was gram positive.

HANGING DROP METHOD

Hanging drop method was carried out to clarify whether the organism was motile or non-motile. The cells were seen at the edges of the coverslip conforming that the microorganism is capable of motility.

STARCH HYDROLYSIS

This test was carried out to confirm the organism was capable of producing

CATALASE TEST

Catalase test was done to make sure that microorganism produced the enzyme catalase. When H_2O_2 was poured onto the petriplate there was a frizzing noise with bubble formation due to the action of catalase which converted H_2O_2 to H_2O and O_2, hence it was confirmed that the microorganism could produce the enzyme Catalase.

CITRATE TEST

The citrate test was done to make sure whether the microorganism was able to use citrate as a sole source of carbon. The green slant slowly turned to blue colour due to the presence of bromthymol blue indicator, confirming that the microorganism could use citrate as its carbon source.

CASEIN HYDROLYSIS

Casein hydrolysis test was performed to clarify whether the microorganism was capable to produce proteolytic enzymes. There was a zone of clearance observed in the skim milk agar confirming that micro organism was actually able to produced proteolytic enzyme.

Thus, the test organism was found to be gram positive, motile, rod shaped, Starch utilizing, citrate utilizing, hydrolytic enzyme and proteolytic enzyme producing organism. It was confirmed that the pure culture sent from IMTECH, Chandigarh was a *Bacillus* species.

TABLE 4.1. RESULT FOR BIOCHEMICAL TESTS

NAME OF BIOCHEMICAL TEST	RESULT
GRAM STAINING	GRAM POSITIVE
HANGING DROP METHOD	MOTILE
STARCH HYDROLYSIS	POSITIVE
CATALASE TEST	POSITIVE
CITRATE TEST	POSITIVE
CASEIN HYDROLYSIS	POSITIVE
TRIPLE SUGAR IRON TEST	NEGAVTIVE

4.2. DISCUSSION

The extraction process from shake flask and bioreactor from day 1 to 10 from a culture of *Bacillus subtilis* in half strength and full strength fermentation medium obtained the crude bacitracin. Identification of the crude bacitracin was done using thin layer chromatography technique where the crude bacitracin was run along with the standard bacitracin (Ali and Janabi., 2006). The results showed that the sample contain some amount of bacitracin. According to the standard procedure 26µg of bacitracin produced 1mm of zone of clearance against *Micrococcus luteus* (Harvey., 1980). This standard was verified by agar ditch diffusion method, the zone of clearance was measured and the antibiotic concentration was calculated accordingly.

For the first two days there was no production of bacitracin observed. From the 3rd day till the 7th day there was an increase in the production of bacitracin. The maximum antibiotic concentration 52.0µg/20µl and the maximum zone of clearance 20mm were observed on the 7th day in full strength medium in bioreactor. The pH changed slightly acidic everyday and reached the maximum of 5.0 on the 10th day.

The production of Bacitracin from *Bacillus licheniformis* through fermentation technology by using defatted oil seed cakes as the starting material was studied very recently (Farzana *et al.*, 2004). The study shows that the

S.NO	DAYS	ANTIBIOTIC CONCENTRATION µg/20µl	pH	ZONE OF INHIBITION (mm)
1	1	-	7.0	-
2	2	-	7.0	-
3	3	15.6	7.0	6
4	4	20.8	7.0	8
5	5	26.0	7.0	10
6	6	28.8	6.7	11
7	7	31.2	6.5	12
8	8	26.0	6.0	10
9	9	23.4	5.5	9
10	10	18.2	5.0	7

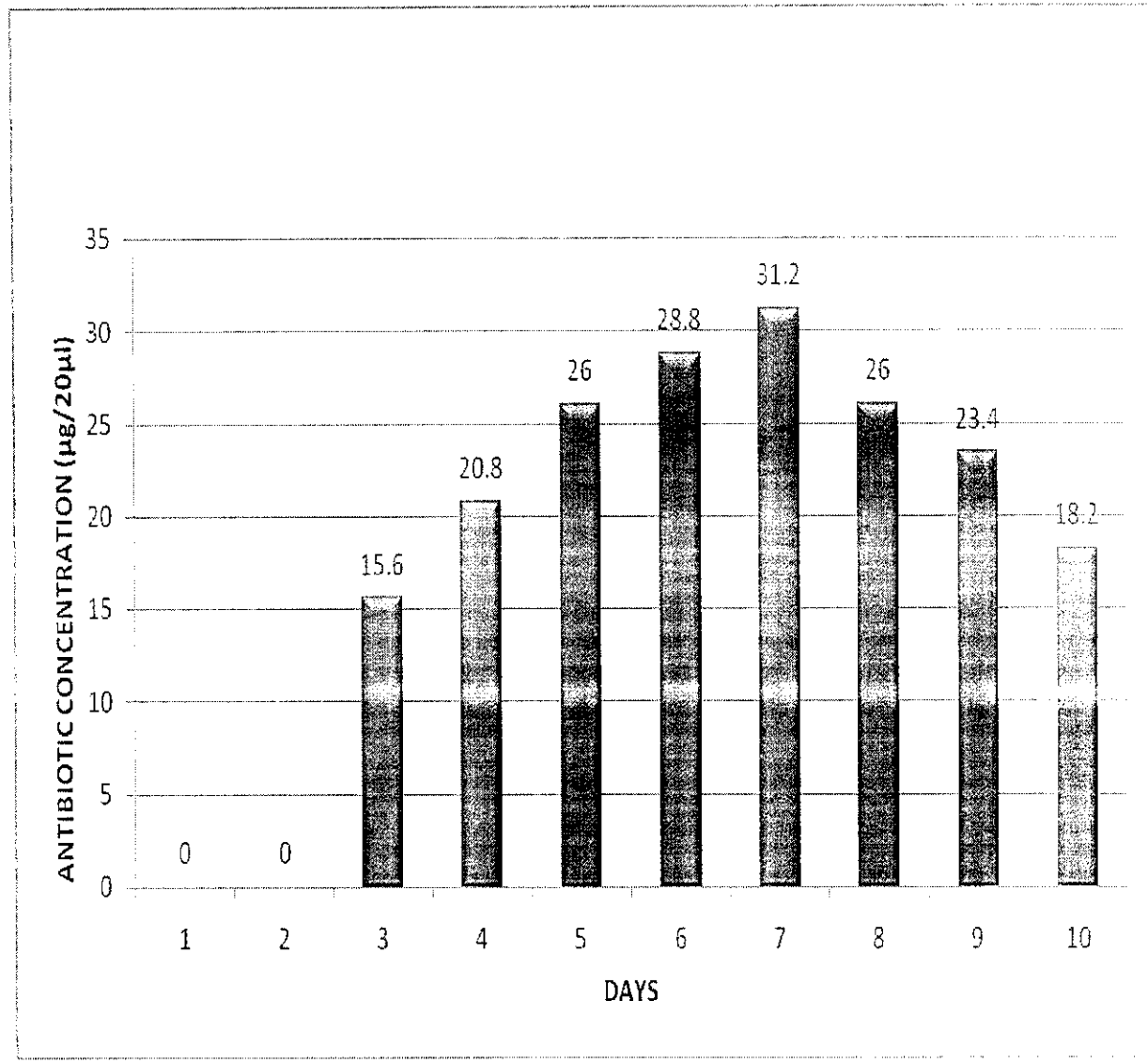
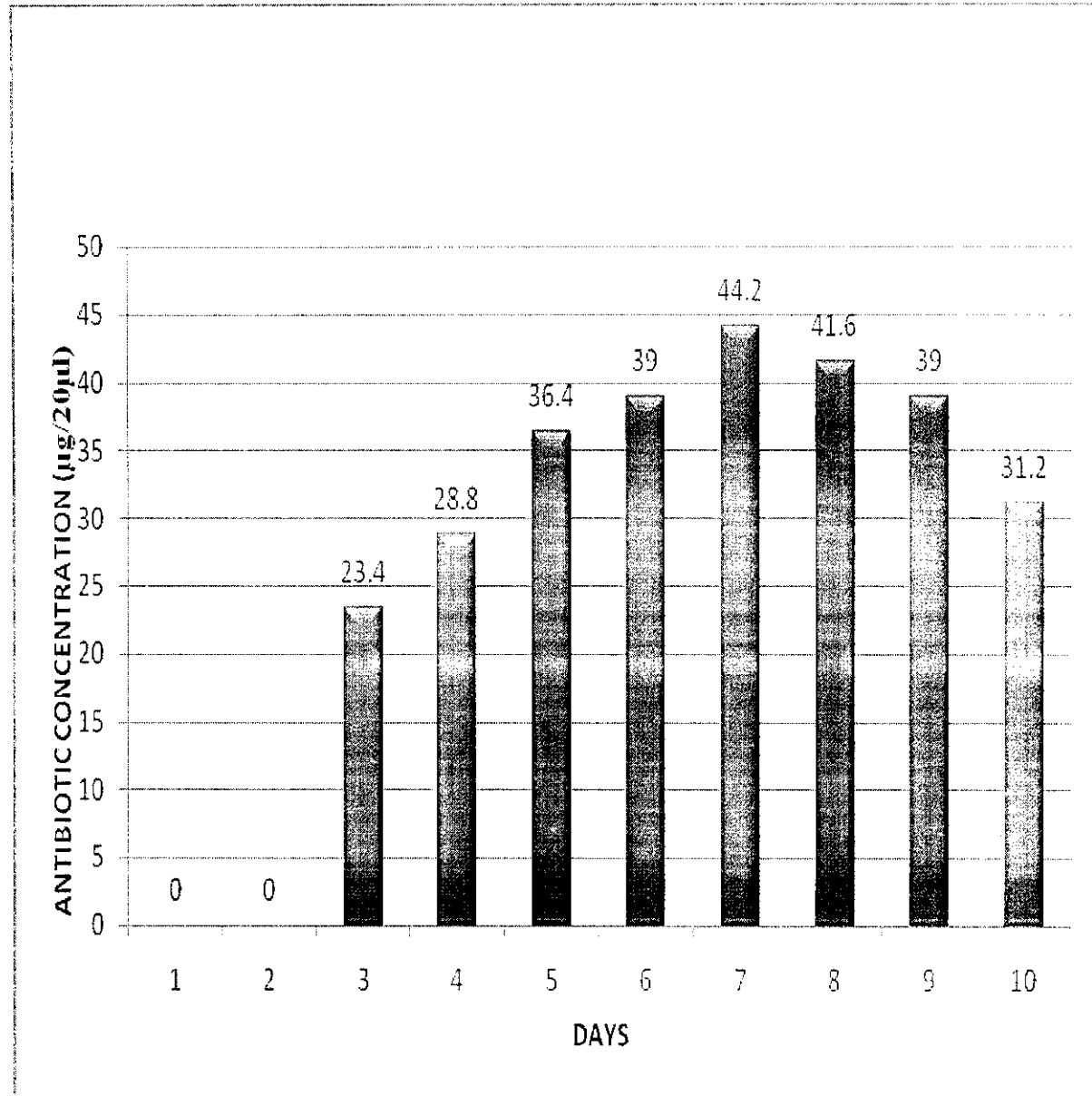
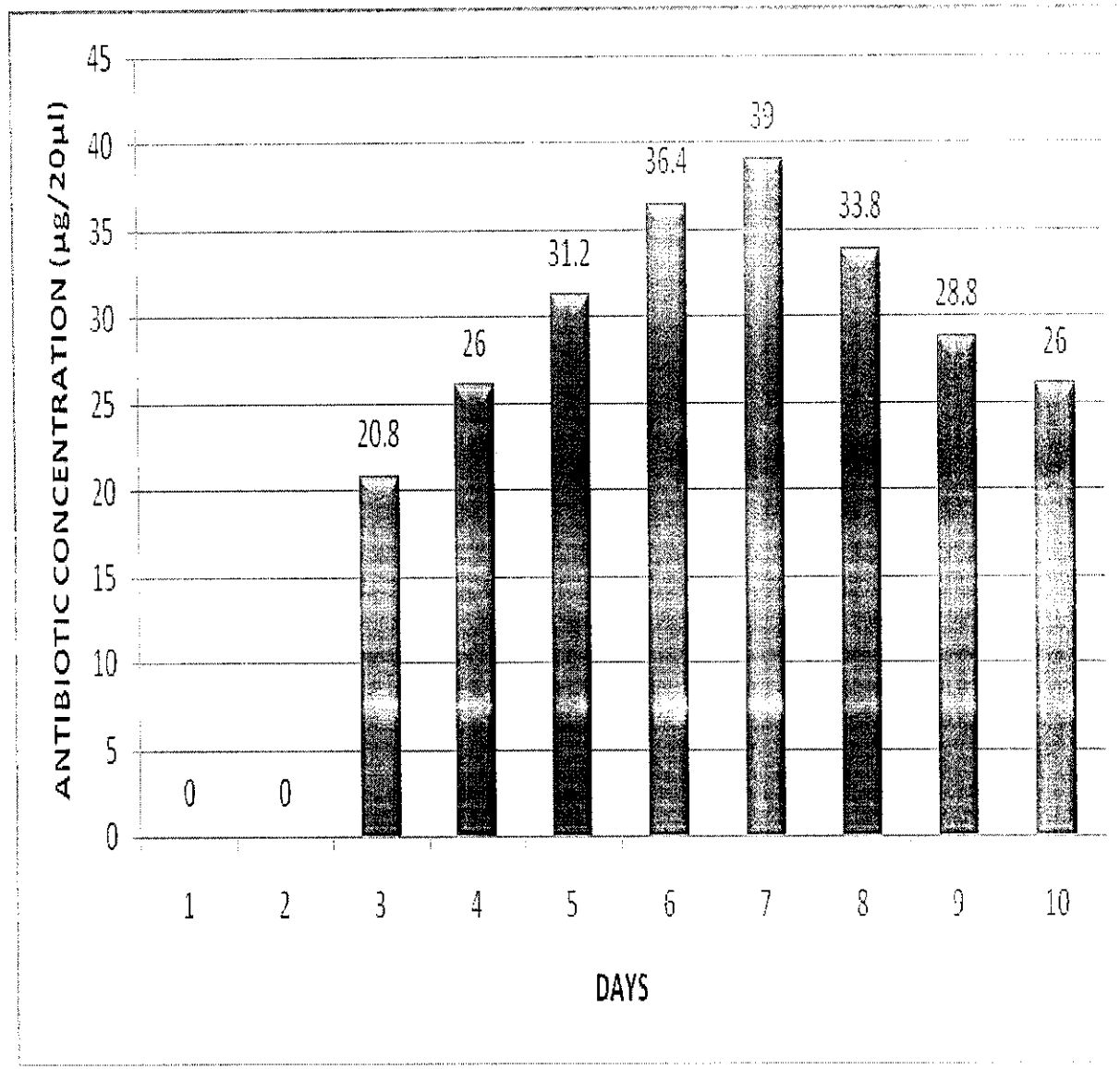


FIGURE 4.2.1 PRODUTION OF BACITRACIN IN HALF STRENGTH

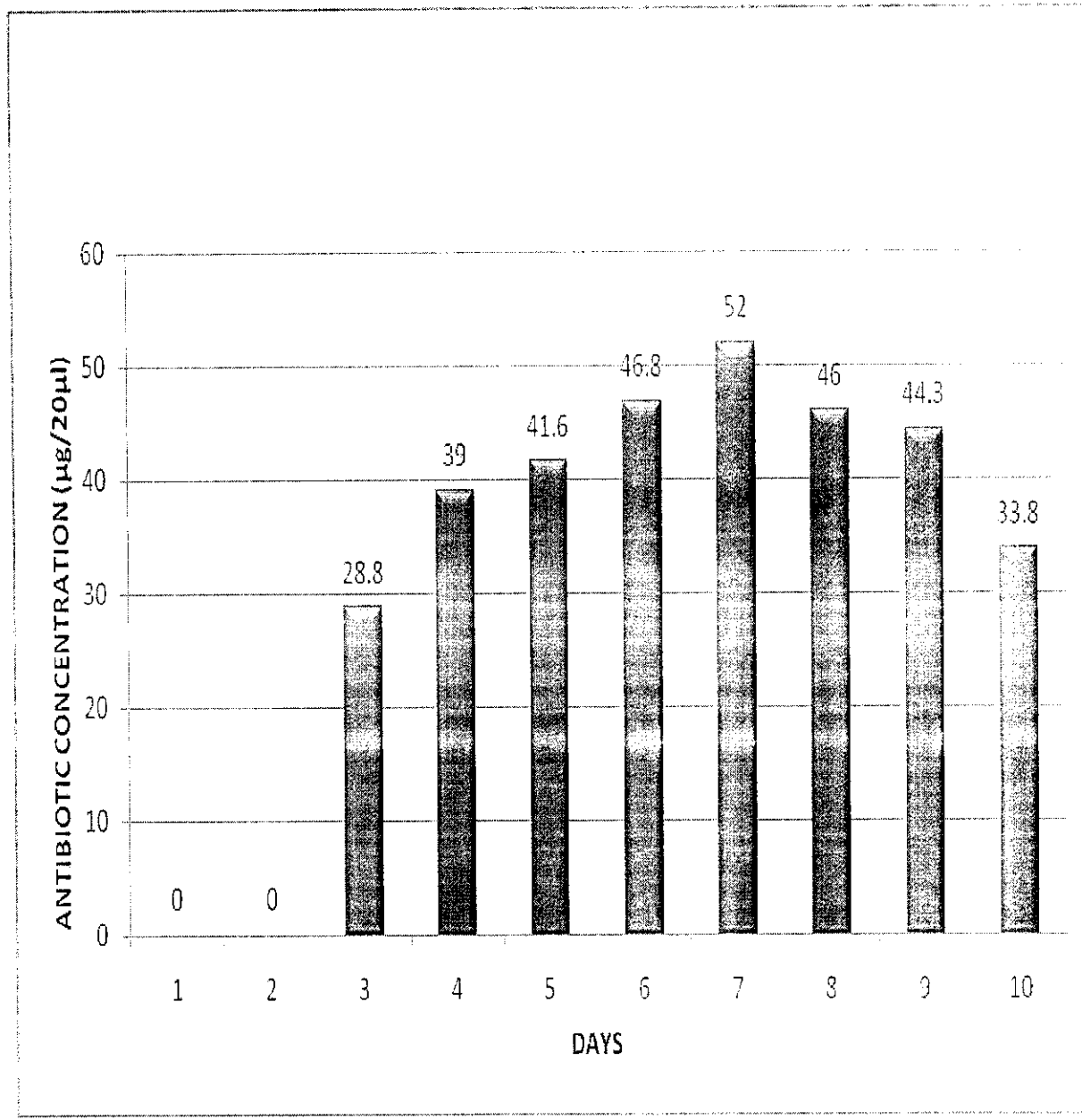
S.NO	DAYS	ANTIBIOTIC CONCENTRATION µg/20µl	pH	ZONE OF INHIBITION (mm)
1	1	-	7.0	-
2	2	-	7.0	-
3	3	23.4	7.3	9
4	4	28.8	7.2	11
5	5	36.4	7.0	14
6	6	39	7.0	15
7	7	44.2	6.4	17
8	8	41.6	6.0	16
9	9	39.0	5.8	15
10	10	31.2	5.4	12


ANTIBIOTIC CONCENTRATION (µg/20µl)
50
45
40
35
30
25
20
15
10
5
0
0
0
23.4
28.8
36.4
39
44.2
41.6
39
31.2
1
2
3
4
5
6
7
8
9
10
DAYS

S.NO	DAYS	ANTIBIOTIC CONCENTRATION µg/20µl	pH	ZONE OF INHIBITION (mm)
1	1	-	7.0	-
2	2	-	7.0	-
3	3	20.8	7.2	8
4	4	26.0	7.2	10
5	5	31.2	6.5	12
6	6	36.4	6.5	14
7	7	39.0	6.5	15
8	8	33.8	6.4	13
9	9	28.8	6.4	11
10	10	26.0	6.0	10


ANTIBIOTIC CONCENTRATION (µg/20µl)
45
40
35
30
25
20
15
10
5
0
0
0
20.8
26
31.2
36.4
39
33.8
28.8
26
1
2
3
4
5
6
7
8
9
10
DAYS

S.NO	DAYS	ANTIBIOTIC CONCENTRATION µg/20µl	pH	ZONE OF INHIBITION (mm)
1	1	-	7.0	-
2	2	-	7.0	-
3	3	28.8	7.2	11
4	4	39.0	7.2	15
5	5	41.6	6.5	16
6	6	46.8	6.5	18
7	7	52.0	6.5	20
8	8	46.0	6.4	18
9	9	44.3	6.4	17
10	10	33.8	6.0	13


ANTIBIOTIC CONCENTRATION (µg/20µl)
60
50
40
30
20
10
0
0
0
28.8
39
41.6
46.8
52
46
44.3
33.8
1
2
3
4
5
6
7
8
9
10
DAYS

The study was carried outwith an objective to show that bacitracin could be effectively produced from *Bacillus subtilis* in a very economic way. *Bacillus subtilis* was obtained from IMTECH, Chandigarh and its genus specificity was verified by various biochemical tests like Gram staining, Hanging drop method, Starch hydrolysis, Catalase test, Citrate test, Triple sugar iron test and Casein hydrolysis.

A 24hours culture of *Bacillus subtilis* was grown in shake flasks and stirred tank fermentor with half strength and full strength medium for 10 days. Everyday crude bacitracin was extracted from 1ml of the fermentation medium. The crude bacitracin was identified by thin layer chromatography and quantified by agar ditch diffusion method. The antimicrobial activity of the crude bacitracin was found out by Kirby-Bauer method against *E.coli* bacteria.

Thus from the tabulated values we have concluded that *Bacillus subtilis* is an effective microorganism that can be used for the production of bacitracin in an economic way.

APPENDIX 1:

SYNTHETIC FERMENTATION MEDIUM:

L- GLUTAMIC ACID	5 grams
KH_2PO_4	0.5 gram
K_2HPO_4	0.5 gram
$MgSO_4.7H_2O$	0.2 gram
$MnSO_4.7H_20$	0.01 gram
NaCl	0.01 gram
$FeSO_4.7H_2O$	0.01 gram
$CUSO_4.7H_2O$	0.01 gram
$CaH_4(PO_4)_2$	2 gram
DISTILLED WATER	1000 ml
pH	7.0

Glucose was sterilized separately and was added according that concentration of sugar finally becomes 1%.

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