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Effects of varied culture conditions on crude bacitracin produced by *Bacillus subtilis* isolated from the soil

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Abstract

Aim: To determine the effects subjecting *Bacillus subtilis* to different cultural conditions will have on the crude Bacitracin produced in inhibiting the growth of known susceptible organisms.

Method: *Bacillus subtilis* known to be a bacitracin producer was isolated from the soil and identified. The isolate was used to produce bacitracin under various culture conditions with peptone water serving as a basal fermentation medium. The resultant crude bacitracin produced was checked for its antimicrobial activity against *Staphylococcus aureus* by using the agar well diffusion method. Culture conditions varied were the addition of glycerol, arabinose, fructose, mannose, sucrose, urea, $MgSO_4$, $CaCO_3$, KCl, sodium citrate, and the adjustment of initial pH at pH 5, 6, 8 and 9. Bacitracin production was done at 37 ^oC for 4 days in an orbital shaker incubator rotating at 150rpm.

Result: The antimicrobial activity against the test organism (*Staphylococcus aureus*) was determined by the mm of the zone of inhibition, with the best result obtained from the addition of glycerol (14 mm) and the least zone of inhibition was observed from the addition of $MgSO_4$ (3 mm). Also, pH 8 produced the best result (20 mm), while at pH 5, the antimicrobial activity of the crude bacitracin was reduced (14 mm).

Conclusion: Bacitracin produced from *Bacillus subtilis* under various culture conditions has antimicrobial effect against *Staphylococcus aureus*. The highest antimicrobial activities can be gotten by the addition of glycerol and by raising the initial pH of the basal broth to pH 8 while that produced by addition of $MgSO_4$ will have the lowest antimicrobial effect.

Keywords: *Bacillus subtilis*; Bacitracin; Culture conditions; Antimicrobial activities; *Staphylococcus aureus*

1. Introduction

Bacillus species are known for their ability to produce different molecular substances with varying degrees of inhibitory effect against specific microorganisms. These molecular substances are called antibiotics, derived from the Greek word “antibiosis” which literally means “against life”. These antibiotics are low molecular weight (non-protein) molecules produced as secondary metabolites mainly by microorganisms found in the soil [1]. They can be purified from microbial fermentation and modified chemically or enzymatically for either chemical use or fundamental studies. *Bacillus subtilis*, a gram-positive, spore-forming, rod-shaped aerobic bacterium usually found in the soil belongs to this genus. They can grow in minimalistic media having only essential nutrients. Their division and growth require the remodelling and

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synthesis of the thick bacteria cell wall with the bacteria possessing a number of regulatory mechanisms [2]. When *Bacillus subtilis* grows in nutrient-deficient conditions, it responds in a number of ways to ensure its survival. One of these responses includes synthesizing antibiotics and extracellular proteins and enzymes. The bacterium may also take up DNA from its environment, develop flagella for motility and form biofilms. Also, *Bacillus subtilis* can form spores in response to extreme environmental and nutritional conditions. These spores are dormant and heat-resistant up to about 95 °C [3]. The spores quickly return to its vegetative state when the temperature becomes more favourable or the nutrient drought is reversed. Aside from being a spore former, *Bacillus subtilis* is known to produce an array of bioactive compounds including antibiotics, lantibiotics, polyketides and many polypeptides, one of which is Bacitracin [4].

Bacitracin is a white to pale buff, usually odourless hygroscopic powder that is freely soluble in water but insoluble in chloroform, acetone and ether. It is a commercially produced antibiotic used topically for the treatment of several dermal infections. It is a polypeptide antibiotic that inhibits the cell wall synthesis of susceptible bacteria, and it acts by altering membrane permeability and inhibiting the formation of peptidoglycan chains present in the cell wall of susceptible bacteria, usually gram-positive [5]. It also has an intramuscular formulation that is administered to infants for the treatment of pneumonia and empyema caused by susceptible staphylococci, although it is less preferred due to a potential nephrotoxicity and the availability of more efficient alternatives [6]. It was first isolated in 1943 by Johnson, Anker and Meleney from the Tracey 1 strain of *Bacillus subtilis* and they called it bacitracin because it was isolated from a young girl named Tracy who had a compound fracture with damaged tissue and street contamination [7]. While Bacitracin is mostly active against many gram-positive bacteria like *Staphylococcus aureus* due to the peculiarities of their cell walls, studies have also shown its inhibitory activity against some known gram-negative bacteria such as *Neisseria spp.*

2. Material and methods

2.1. Sample Collection

The soil sample used for the experiment was aseptically collected from the undergraduate SIWES (Students' Industrial Work Experience Scheme) farm of Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria. Following collection, the sample was transported to the Microbiology laboratory and weighed.

2.2. Screening for Bacillus subtilis

1g of soil sample was transferred into a screw cap test tube containing 9 ml of sterile distilled water to create a 10^{-1} dilution. The test tube was agitated severally after which it was placed in a water bath and heated to 70°C for 10 minutes. The purpose of this was to eliminate mesophilic organisms and other non-spore formers that could be present in the soil sample.

Serial dilution was done by transferring 1 ml of soil and water solution into 9 ml of sterile distilled water to create a 10^{-2} dilution. This was done with the use of sterile 1ml pipettes. From the 10^{-2} dilution, 1 ml was taken and transferred into another tube containing 9 ml of sterile distilled water to create a 10^{-3} dilution. This process was repeated until a 10^{-6} dilution was achieved.

1.0 ml of 10^{-1}, 10^{-3} and 10^{-6} dilutions were inoculated using the pour plate method. The inoculum was first transferred aseptically into Petri plates after which approximately 20ml of sterilized nutrient agar prepared according to the manufacturer's instructions and cooled to 45 °C were poured into the plates, swirled gently and allowed to solidify. The plates were inverted and incubated at 37 °C for 24 hours.

2.3. Morphological Characterization, Gram Reaction and Endospore Staining

Macroscopic examinations of the incubated plates were carried out to determine the cultural characteristics such as colony size, shape, colon, elevation, margin and texture. The distinct colonies were then assigned unique codes with their cultural characteristics recorded. Afterwards, the gram staining technique was used to screen out unwanted microorganisms by their gram reaction. The procedure was carried out, according to Cheesborough [8] as follows: a smear was made on a clean, grease-free labelled glass slide containing a drop of normal saline and was left to air dry. It was thereafter heat-fixed by rapidly passing it over a Bunsen burner three times. After cooling, the smear was flooded with crystal violet solution for 60 seconds and then washed indirectly with clean, running tap water. It was tipped off all the water before being flooded with Lugol's iodine solution (mordant) for 60 seconds. It was then washed off indirectly with clean tap water. Acetone alcohol was used to rapidly decolourize the smear for about 15 seconds before being washed in an indirect flow of clean tap water. Finally, Safranin was used to cover the smear for two minutes and washed away with an indirect flow of clean tap water. The back of the slide was wiped clean and placed in a draining

rack to air dry. After air drying, a drop of immersion oil was added and examined under the microscope with the x100 oil immersion objective. Following microscopic examination, isolates which were found to be gram-positive rods were subjected to the endospore staining technique while the rest were eliminated.

Endospore staining was carried out using the Schaeffer-Fulton method as outlined by Hussey and Zaiyatz [9] in the Endospore Staining Protocol of the American Society of Microbiology. The procedure was carried out as follows: a smear of the gram positive, rod shaped colonies was prepared on a labelled clean glass slide containing a drop of sterile distilled water and allowed to air dry. It was then heat fixed and covered with a square cut out blotting paper that fitted the slide. The blotting paper was consistently saturated with malachite green stain solution over a container of boiling water for 5 minutes. It was thereafter washed gently with clean tap water. The slide was then covered with safranin for 30 seconds after which it was rinsed with clean tap water and blotted dry. A drop of immersion oil was applied to the slide and viewed under the x100 oil immersion objective lens.

2.4. Biochemical Tests

The following biochemical tests were conducted on colonies which were gram-positive, rod-shaped and possessed endospores: motility test, catalase test, citrate test, oxidase test, indole test, gelatin hydrolysis, methyl red/ voges proskauer, and sugar fermentation tests. These tests were carried out using the standard procedures outlined by Cheesbrough [8], McDevitt [10], dela Cruz and Torres [11] and Reiner [12]. Following these tests, Bergey's manual of determinative bacteriology [13] was used to identify *Bacillus subtilis* from other species of Bacillus present. The isolate was then inoculated in nutrient broths for further studies and as well stored in a slant as stock.

2.5. Screening for Test Organism

The test organism used for this research was *Staphylococcus aureus*. The bacterium was isolated from the human nasal cavity. Sterile swab sticks were used to collect nasal swabs. They were then agitated into 9ml of sterile water. From this dilution, 1 ml was inoculated in Mannitol Salt Agar using the spread plate method. Colonies indicative of *Staphylococcus aureus* were sub-cultured into nutrient agar from where they were gram-stained and tested for motility, catalase, citrate utilization, oxidase, indole, gelatin hydrolysis, coagulase, methyl red/ voges proskauer and sugar fermentation tests [8, 10, 11 and 12]. *Staphylococcus aureus* was identified using Bergey's manual of determinative bacteriology [13]. Also, bacitracin susceptibility test was carried out to determine its susceptibility to Bacitracin. The procedure used is outlined by Baron and Finegold [14].

2.6. Preparation of Selected Culture Parameters

Following the isolation of *Bacillus subtilis*, the organism was transferred into basal media containing various culture materials. The basal media used for this experiment was peptone water [15] prepared according to the manufacturer's instructions. The selected culture parameters were the addition of Arabinose, Glucose, Fructose, Mannose, Sucrose, Glycerol, Urea, Calcium carbonate ($CaCO_3$), Sodium Citrate, Magnesium sulphate ($MgSO_4$) and Potassium Chloride (KCl), pH ranges of 5, 6, 8, 9 and an unaltered condition comprising only of peptone water.

0.35g (1%) of each substrate was added into 125ml Erlenmeyer flasks containing 35ml of peptone water and labelled accordingly. pH ranges were adjusted with acetic acid and sodium hydroxide to an accuracy of ± 0.2. The bottles were autoclaved at 121^{o} C for 15 minutes. They were then allowed to cool before inoculation with 1.0ml of a 24-hour-old nutrient broth culture of *Bacillus subtilis*.

Afterwards, they were incubated in an orbital shaker incubator at 150 revolutions per minute (rpm) for 4 days at 37^{O}C. At the completion of the 4th day of incubation, they were centrifuged at 5,000 rpm for 15 minutes. The supernatants containing crude bacitracin were collected in labelled sterile containers.

2.7. Challenging Test Organism with Crude Bacitracin

0.1 ml of 24 hours old nutrient broth culture of *Staphylococcus aureus* was spread across the surface of Mueller-Hinton Agar plates prepared according to the manufacturer's instruction. The spread plates were aseptically punched at 5 separate locations using sterile tips of 6mm diameters [16]. 0.1ml of 1, 1:2, 1:4, 1:8 and 1:16 concentrations of the crude bacitracin extracts were added into each of the wells and incubated at 37 ^{o}C for 24 hours. The concentrations were obtained by diluting the extract with sterile distilled water. The diameters of the zone of inhibition were measured in mm and recorded.

3. Results

3.1. Morphological Identification, Biochemical Identification, Gram and Spore Stain Reactions of Bacterial Isolates

Table 1 shows the Morphological identification, Biochemical Identification, Gram and Spore stain reaction of bacterial isolates used for this study. The bacterial isolates were *Bacillus subtilis* and *Staphylococcus aureus.*

Table 1 Identification of Bacteria Isolates

COL	GR	MOR	ES	MOT	OX	CA	CI	MR	VP	IN	CO	GH	LA	XY	GA	Organism
On nutrient agar; they appeared as rough, opaque slightly yellow colonies with jagged edges	+ve	Rods appearing in short chains	Bright green ellipsoidal, central, paracentral and subterminal spores. Not swelling the sporiingia	+ve	+ve	+ve	+ve	-ve	+ve	-ve	N/A	+ve	-ve	+ve	+ve	*Bacillus subtilis*
On Mannitol salt agar, they appear as yellow colonies with yellow zones	+ve	Spherical, appearing in clusters	N/A	-ve	-ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve	*Staphylococcus aureus*

Key: COL = Colony, GR = Gram Reaction, MOR = Morphology, ES = Endospore Stain, MOT = Motility, OX = Oxidase, CA = Catalase, CI = Citrate, MR = Methyl Red, VR = Voges Proskauer, IN = Indole, CO = Coagulase, GH = Gelatine Hydrolysis, LA = Lactose, XY = Xylose, GA = Galactose.

3.2. Zones of Inhibition of Crude Bacitracin against Test Organism

Table 2 Zones of inhibition against *Staphylococcus aureus*

Condition (0.35g) (1%)	1	1:2	1:4	1:8	1:16
Arabinose	9	5	3	0	0
Glucose	12	7	5	1	0
Fructose	9	4	2	0	0
Mannose	8	5	2	0	0
Sucrose	7	5	3	0	0
Glycerol	14	8	6	3	1
Urea	12	7	4	1	0
pH 5	14	9	6	2	0
pH 6	15	10	7	3	1
pH 8	20	14	10	4	2
pH 9	18	12	8	3	1
$MgSO_4$	3	1	0	0	0
$CaCO_3$	14	9	6	3	1
Potassium chloride	8	5	2	0	0
Sodium Citrate	8	4	2	0	0
Unaltered	8	4	2	0	0

Table 2 shows the zones of inhibition of crude bacitracin against *Staphylococcus aureus.* The zones are measured in millimetres (mm) and capture the different concentrations of the antibiotic. The highest zone of inhibition for *S. aureus* was 20mm at 100% concentration obtained from adjusting to pH 8 while the least inhibition was 3 mm from the addition of magnesium sulphate.

3.3. Effects of Carbon and Nitrogen Sources and pH on Crude Bacitracin

Figure 1 indicates the effects the different carbon and nitrogen sources had on the crude bacitracin produced as against the absence of it. This effect is determined by the diameter of the zone of clearance of test organisms measured in millimetres (mm). Glycerol was the better of the carbon and nitrogen sources, giving the result of 14 mm zone of inhibition while the least inhibition was obtained from sucrose which resulted in a 7 mm zone of inhibition.

Figure 2 shows the effect initial pH conditions had on the crude bacitracin produced. This was measured by their performance against *S. aureus*. Adjusting the initial pH of the fermentation broth to 8 resulted in the best inhibition zone at 20 mm. pH 5, on the other hand, had the least impact with a diameter of 14 mm on *S. aureus*.

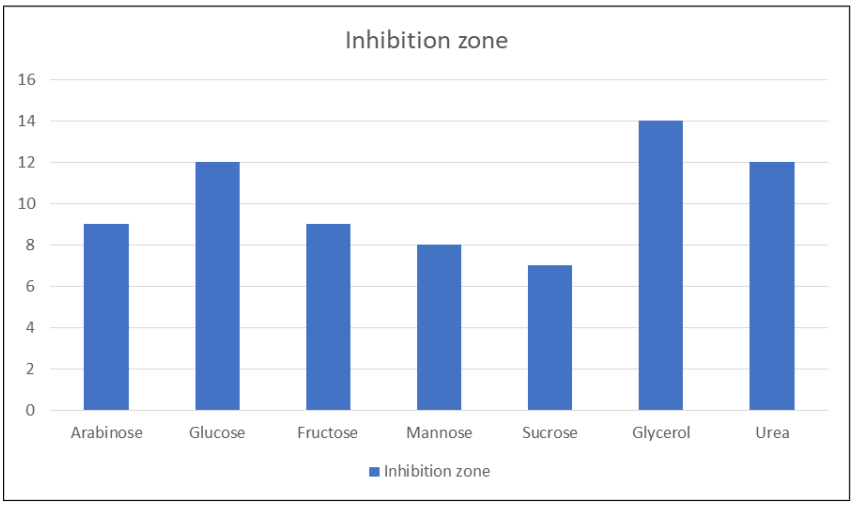

Figure 1 Effects of different carbon and nitrogen sources on crude bacitracin against selected organisms at 100% concentration

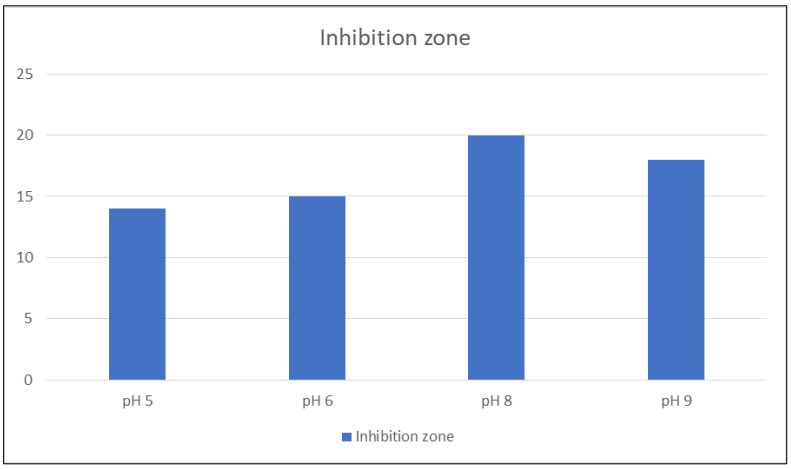

Figure 2 Effects of initial pH on crude bacitracin against selected organisms at 100% concentration

4. Discussion

Several kinds of soil bacteria and fungi are well-known for their ability to produce antibiotics. This special ability could be regarded as a mechanism of survival for the organism whereby they eliminate competition and successfully colonize their desired niches. Many antibiotic-producing soil bacteria are found in the genera Bacillus, Streptomyces and Pseudomonas which are known to produce several medically and agriculturally important antibiotics. The genus *Bacillus* produces a wide variety of antibiotics including bacitracin, polymyxin and colistin, usually during the early stages of sporulation. Predominant among this genus is the rhizobacterium *Bacillus subtilis,* an endospore-forming bacterium that is well distributed in the soil with an average of about 10 cells per gram [4]. The antibiotic-producing capability (Bacitracin) of this bacterium was first discovered in 1943 and is known for its inhibitory activity against many gram-positive and a few gram-negative bacteria.

The present study was carried out to determine the effects varying culture parameters have on the inhibitory activity of crude Bacitracin produced by *Bacillus subtilis.* The impurified, crude bacitracin extract used for this study is justifiable as similar studies have shown that supernatants of *Bacillus subtilis* fermentation contain Bacitracin antibiotics with inhibitory capabilities [7, 17]. Also, *Staphylococcus aureus* was used as the test organism because of its known susceptibility to Bacitracin [18], and the incubation period of 37^{o}C for 4 days used for the production of Bacitracin was due to early reports by Berdy [19] where maximum titres were obtained at 37^{o}C after 3-5 days.

In this study, six different carbon sources were utilized to determine the best carbon source. Maximum inhibitory activity was obtained when glycerol was used as a carbon source (14mm) (Figure 1). Although considerably good activity was obtained when glucose, arabinose and fructose were used as carbon sources (12mm, 9mm and 9mm respectively), Mannose and Sucrose produced lesser effects (8mm and 7mm respectively). In a similar study done by [17], sucrose produced the least inhibitory effect against *S. aureus* with glucose performing better than fructose. Meanwhile, studies involving *Bacillus licheniformis* have reported a markedly increased antibacterial effect when glycerol was used as a carbon source in the fermentation medium [20].

External pH affects several cellular processes including the regulation of the biosynthesis of secondary metabolites. This theme was repeated in this study, as alterations of the initial pH of the fermentation broth produced varying results in the inhibitory activity of the bacitracin produced. The effects at pH 5, 6, 8 and 9 were observed (Figure 2). Among the pH used, pH 5 produced the least inhibitory effect with 14mm at 100% concentration while pH 8 produced the best result with a 20 mm zone of clearance against *S. aureus.* pH 9 resulted in 18 mm clearance while pH 6 was a millimetre above pH 5 with 15 mm. This result is consistent with other studies involving both *Bacillus subtilis* and *Bacillus licheniformis.* The optimum inhibitory activity is usually observed between pH 7 and 9, with pH 8 usually producing the best activity [17, 21, 22, 23, 24].

Urea was the only nitrogen source used for this study. However, it showed a significant enhancing effect (12 mm) on the crude bacitracin inhibitory effects compared to the absence of any additional culture materials (8 mm). Meanwhile, the results from this study show that urea could serve as an equal substitute to glucose (12 mm) and is more effective in enhancing bacitracin production than arabinose, fructose, mannose and sucrose. Shaaban *et al.,* [25] in 2015 showed, through their study, the importance of nitrogen sources on the antibiotic activity of *Bacillus subtilis.*

This study reemphasizes the importance of calcium carbonate ($CaCO_3$) in the production of Bacitracin. A zone of 14mm at 100% concentration against *S. aureus* was observed. Since the commercial production of bacitracin, $CaCO_3$ has always been added to the fermentation broth [26]. The effects of the addition of $CaCO_3$ (14 mm) as compared with the result obtained from the absence of it (8 mm) show the significance of the chemical compound and the role it plays in the production of bacitracin.

In contrast, magnesium sulphate ($MgSO_4$) significantly reduced the production of bacitracin, producing the least inhibition zone of 3 mm. This was particularly strange considering how they are an important component in the fermentation media for bacitracin production [17, 23, 24]. However, studies by Haddar *et al.,* in 2007 reported that increasing amounts of $MgSO_4$ limited the secretion of bacitracin; thus, explaining the result obtained from this study. More so, potassium chloride (KCl) and sodium citrate have been used in the past in the production of bacitracin [27] although the results obtained from this study show that their effect was not as significant (8 mm for KCl and 9 mm for sodium citrate).

5. Conclusion

Bacillus subtilis was isolated from the soil and screened for the production of crude bacitracin. Crude bacitracin was produced using peptone water as the basal medium alongside various alterations to the culture condition. For each altered condition, the crude bacitracin produced was tested for its antimicrobial activity against *Staphylococcus aureus* isolated from the human nasal cavity. This antimicrobial activity was determined by measuring the diameter of inhibition using the agar well diffusion method. The best result was obtained from raising the initial pH of the basal broth to pH 8 while addition of $MgSO_4$ in the medium limits bacitracin secretion. The non-pathogenicity of *Bacillus subtilis* makes the soil bacterium a good candidate for genetic manipulation and commercial production of bacitracin.

Compliance with ethical standards

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Disclosure of conflict of interest

No competing interests exist.

Authors Contributions

This work was carried out in collaboration between the authors, Authors SCI, PCI and CN designed the study, and wrote the protocol. Authors OSE and ROU wrote the first draft of the manuscript. Authors TOO, HON, AKA, AOC, CA and PCC helped with the analysis of the work. The final manuscript was read and approved by the authors.

References

[1] Kumar, D. and Kumar, S. Antimicrobial metabolites and antibiotics obtained from different environmental sources. International Journal of Pharmaceutical Research & Allied Sciences. 2016; 5(3): 85-90.

[2] Piggot, P.J. Bacillus subtilis. In M. Schaechter (Ed), Encyclopedia of Microbiology (Third Edition), (paper 45-56). Cambridge, Massachusetts: Academic Press. 2009.

[3] Janstová B. & Lukásová, J. Heat Resistance of Bacillus spp. Spores Isolated from Cow's Milk and Farm Environment. Acta Veterinaria Brunensis. 2001; 179-184.

[4] Stein, T. Bacillus subtilis antibiotics: structures, syntheses and specific functions. Molecular Microbiology. 2005; 56(4):845-57.

[5] Stone, K.J. & Strominger, J.L. Mechanism of action of bacitracin: complexation with metal ion and C 55 -isoprenyl pyrophosphate. Proceedings of the National Academy of Sciences, U.S.A. 1971; 68(12):3223-7.

[6] Nguyen, R., Khanna, N. R., Safadi, A. O., Sun, Y. Bacitracin Topical. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing. 2021.

[7] Johnson, B. A., Anker, H. & Meleney, F. L. Bacitracin: A new antibiotic produced by a member of the B. subtilis group. Science. 1945; 102 (2650):376-7.

[8] Cheesbrough, M. District Laboratory Practice in Tropical Countries, Part 2. Cambridge University Press. 2006.

[9] Hussey, M. A. & Zayaitz, A. Endospore Stain Protocol – ASM. Available at: https://asm.org/Protocols/Endospore-Stain-Protocol. 2007.

[10] McDevitt, S. Methyl Red and Voges-Proskauer Test Protocols – ASM. Available at: https://asm.org/Protocols/Methyl-Red-and-Voges-Proskauer-Test-Protocols. 2009.

[11] dela Cruz, T. E. E. & Torres, J. M. O. Gelatin Hydrolysis Test Protocol – ASM. Available at: https://asm.org/Protocols/Gelatin-Hydrolysis-Test-Protocol. 2012.

[12] Reiner, K. Carbohydrate fermentation protocol – ASM. Available at: https://asm.org/Protocols/Carbohydrate-Fermentation-Protocol. 2012

[13] Bergey, D.H. & Holt, J.G. Bergey's manual of determinative bacteriology. Baltimore: Williams & Wilkins. 1994.

[14] Baron, E. J. & Finegold, S. M. Bailey & Scott's Diagnostic Microbiology (8th Edition). St. Louis: Mosby. 1990.

[15] Morikawa, Y., Ochiai, K., Karine, I. & Suzuki, S. Bacitracin Production by Whole Cells Immobilized in Polyacrylamide Gel. Antimicrobial Agents and Chemotherapy. 1979; 15(1): 126–130.

[16] Balouiri, M., Sadiki, M., Ibnsouda, S.K. Methods for in vitro evaluating antimicrobial activity: A review. Journal of Pharmaceutical Analysis. 2016; 6(2):71-79.

[17] Rukmini, M., Sahoo, D., Dalei, J. & Ray, R. Production, purification and characterization of bacitracin from Bacillus subtilis. The Pharma Innovation Journal. 2015; 3(12): 77-82.

[18] Kumar, P. Pharmacology of Specific Drug Groups: Antibiotic Therapy. In F. J. Dowd, B. S. Johnson, A. J. Mariotti (Eds), Pharmacology and Therapeutics for Dentistry (Seventh Edition) (paper 457-487). London: Mosby. 2017.

[19] Berdy, J. Recent developments of antibiotic research and classification of antibiotics according to chemical structure. Advances in applied Microbiology. 1974; 18: 309-406.

[20] Haddar, H. O., Aziz, G. M. & Al-Gelawi, M. J. Optimization of Bacitracin production by Bacillus licheniformis B5. Pakistan Journal of Biological Sciences. 2007; 10(6): 972-976.

[21] Snoke J.E. Formation of Bacitracin by Washed Cell Suspensions of Bacillus licheniformis. Journal of Bacteriology. 1960; 80(4): 552-557.

[22] Hanlon, G.W. & Hodges, N. A. Bacitracin and protease production in relation to sporulation during exponential growth of Bacillus licheniformis on poorly utilized carbon and nitrogen sources. Journal of Bacteriology. 1981; 147: 427-431.

[23] Awais, M., Pervez, A., Yaqub, A. & Shah, M. M. Production of Antimicrobial Metabolites by Bacillus subtilis Immobilized in Polyacrylamide Gel. Pakistan Journal of Zoology. 2010; 42(3): 267-275.

[24] Gulhane, P. A., Gomashe, A. V. & Lade, S. Optimization of Bacitracin production from bacillus licheniformis NCIM 2536. International Journal of Current Microbiology and Applied Sciences. 2014; 3 (9): 819-829.

[25] Shaaban, M. T., Attia, M., Mowafy, E. I., Turky, A. S. & Awad, N. M. Identification and Improving Antibiotic Production by Some Bacteria Isolated from Egyptian Soil. Global Advanced Research Journal of Agricultural Science. 2015; 4(9): 517-532.

[26] Ziffer, J. & Wis, M. Production of Bacitracin. U.S. Patent No. 2,813,061. Washington, DC: U.S. Patent and Trademark Office. 1957.

[27] Haavik, H. I. Studies on the Formation of Bacitracin by Bacillus licheniformis: Effect of Inorganic Phosphate. Journal of General Microbiology. 1974; 84: 226-230.