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Production, purification and characterization of bacitracin from *Bacillus subtilis.*

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Abstract
Bacillus subtilis capable of producing bacitracin was isolated from soil and screened for the production of bacitracin on nutrient media against test pathogenic organisms viz: *Micrococcus luteus,* (ATCC 9341) and *Staphylococcus aureus* (ATCC 13565). Bacitracin was produced by submerged fermentation from the isolated bacteria and checked for their antimicrobial activity by agar well diffusion method. The bacitracin was extracted and purified by Butanol-Ether liquid extraction system and identified by thin layer chromatography. By optimizing the media composition and culture conditions, the effect of different nitrogen sources (Asparagine, Methionine, peptone, yeast extract, Sodium nitrite, Ammonium nitrite), carbon sources (glucose, fructose, sucrose, lactose) pH (5, 6, 7, 8, 9), time (48 hours, 72 hours, 120 hours, 144 hours), temperature (-4 ˚C, 37 °C, 42 °C and 30 °C) on the production of bacitracin were investigated by agar diffusion assay as detected by the size of zones of inhibition. The maximum production was observed by using glucose as carbon and asparagine as nitrogen source and after incubating at 42 °C of pH 7.0 for 144 hours.

Keywords: Bacitracin, *Bacillus subtilis*, Antimicrobial activity

1. Introduction
The discovery and use of 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 [1] and these compounds can fight against various diseases. Generally antibiotic is a chemical substance, possessing a molecular weight lesser than 2 kilo Dalton and used to kill or prevent growth of any other type of microorganisms at a lower dosase [2].
Most of the peptide antibiotics are produced by bacilli that 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 [3]. Bacitracin is derived from cultures of *Bacillus subtilis*. It is a white to pale buff, hygroscopic powder, odorless or having a slight odor. It is precipitated from its solutions and inactivated by many of the heavy metals. Bacitracin is a mixture of several polypeptides differing in their amino acid composition [4] and functions as an inhibitor of cell wall biosynthesis. Bacitracin of other micro-organism is an antibiotic as well as non-ribosomally produced by *Bacillus licheniformis* [5]. Bacitracin affects protein synthesis, cell wall synthesis and membrane functions. Studies on antibiotics and antibiotics with enzyme combination have been made by Neeraj *et al*, 2010 [6] and Meshcer, 1974 [7].
It is a potent antibiotic directed primarily against gram positive organisms like *Bacilli spp*, *Staphylococcus* sp. *Streptococcus* sp. *Clostridium* sp. as well as some Archaebacteria [8]. It is one of the important antibiotic used in human medicine and also used in animal husbandry for the prevention and control of diseases existing in farm animals.
It is an in gradient in several commercially available topical triple antibiotic ointments such as Polysporin and Neosporin that are used to prevent infections in minor cuts and burns **[9]**. Bacitracin is an important feed supplement for a number of animal species. It improves the weight gain and feed efficiency when added in a concentration of 5-100 ppm.

2. Materials and Methods
2.1 Isolation and screening of Bacillus species
Soil samples were collected in zipper pouch from rhizosphere and subjected to 10 fold serial dilution and aliquots (0.1 ml) was plated on Nutrient agar plates and incubated for 24 hours at optimal conditions [10]. The isolated strains were kept in slant cultures at 4 °C.

2.2 Characterization of isolated strains
Isolated strains were identified on the basis of their morphological and biochemical characteristics like gram staining, endospore staining, starch hydrolysis test, MR-VP test, motility test, citrate test, 6.5% NaCl test etc. according to Bergey's Manual of Determinative Bacteriology [10].

2.3 Screening of Bacitracin production
The production of bacitracin by the isolated bacteria was confirmed on test organisms to be screened. Test organisms were streaked as zig-zag fashion on nutrient agar media on which a central streak was done. The result was taken as positive by the creation of zone of inhibition on the side of the streak.

2.4 Submerged fermentation for production of Bacitracin
The submerged fermentation was carried out with synthetic media in 250 ml conical flask for the production of bacitracin. Synthetic media containing the following composition in (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; $CaCl_2.2H_2O$-0.015; Glucose-10; pH-7 was prepared and autoclaved for 15 minutes at 121 ^{0}C at 15 psi pressure . After cooling, the media were inoculated with 24 hours old vegetative culture (inoculums) and the flask was incubated at 30 °C in an orbital shaker at 150 rpm. After 48 hours of incubation, the fermentation broth was centrifuged at 10,000 rpm for 15 minutes and supernatants were assayed for antimicrobial potency.

2.5 Purification of antibiotic by solvent extraction method
An equal volume of Butanol-Ether solution was added to aqueous concentrate in a separating funnel and shaken vigorously. Concentrated HCl is added drop by drop until the mixture is separated into layers immediately after shaking. The upper layer becomes darker as acid is added owing to the removal of impurities from the aqueous chamber. The pH of aqueous layer is maintained between 3 - 4. The lower aqueous layer which contains the active material is decanted and the extraction is repeated. The extraction is repeated five times allowed to stand for ten minutes and pH is tested. The lower layer is decanted and extracted five times with peroxide-free ether. 40 ml of the extract was obtained from submerged fermentation. The final aqueous layer is distilled under reduced pressure until all butanol and ether has been removed. The volume of the extracts is 13 ml for submerged. After distillation the solution is brought to pH 7 with sodium bicarbonate. The neutralized solution is then lyophilized, which is a yellowish powder. 100 mg of MgO is added to 10 ml of faintly acidic or neutral solution of concentrated bacitracin. This mixture is stirred and after several hours storage in the refrigerator it is filtered cold. The filtrate was found alkaline and was neutralized with HCl and MgO treatment. The process was repeated until the activity of MgO is negligible. The first formed precipitate was discarded as it does not exhibit antibiotic activity. The filtrate was neutralized with sodium bicarbonate and salicylic acid was then added for complete precipitation. The precipitate was collected and dried, repeatedly washed with peroxide free ether to remove excess of salicylic acid. It is grounded in funnel and washed again with peroxide-free ether, dried in vacuum at room temperature over phosphoric anhydride. The salicylate was dissolved by percolating 0.05 N HCl and used for assay.

2.6 Agar diffusion assay
Agar well diffusion method was used to check the cultures for the production of antimicrobial metabolites [11]. 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 standards (106 cfu/ml). 200 µL of standardized cell suspensions were spread on a Mueller-Hinton agar. Wells were then bored into the agar using a sterile 6 mm diameter cork borer. Crude extracts of different conc. (70 µl, 80 µl, 90 µl, 100 µl) were introduced into the wells, allowed to stand at room temperature for about 2 hours and then incubated at 37 °C. After 24 hrs of incubation the zones of inhibition were observed and measured.

2.7 Optimum condition for antibiotic production
Optimization of culture conditions and media composition was done to study the effects on the productivity of antibiotic bacitracin. It was done by studying the effects of different carbon sources(Glucose, sucrose, fructose and lactose), nitrogen sources (Asparagine, Methionine, Yeast extract, Peptone, Sodium nitrite and ammonium nitrite) and changing the pH (5, 6, 7, 8, 9) incubation time (48 hr, 72 hr, 120 hr, 144 hr) and incubation temperature(-4 °C, 37 °C, 42 °C, room temp 30 °C).

2.8 Identification of Bacitracin using thin layer chromatography
Silica gel was prepared with distilled water in 1:2 ratio and immediately transferred over the glass slide uniformly, air dried and kept in the oven at 105 °C for 30 min. The solvent for mobile phase was prepared as Butanol: Water (8:2). The reference line was drawn on the silica gel plate at a distance of 1.5 cm from the lower end. Spots were marked on the end and the test samples were applied on the spots (10-20 µl) the silica gel plate was suspended in the solvent. The plate was taken out when the migration of the solvent, nearly equals to 80% of the length of the plate. The characterization was done by comparing the retardation factor of bacitracin to the standard bacitracin retardation factor.

Retardation factor (R_f) = distance travelled by solvent / distance travelled by solute

3. Results and Discussion
3.1 Isolation and identification of microorganisms
Well grown colonies were selected and screened for bacitracin production. Four colonies were identified on the basis of morphology study, gram staining and biochemical tests (Table 1). After performing all the tests colony 1, 2, 3 and 4 were confirmed as *Bacillus subtilis, Bacillus licheniformis, Bacillus cereus* and *Bacillus polymyxa* respectively.

Table 1: Biochemical tests for *Bacillus spp*.

Biochemical tests	Colony 1	Colony 2	Colony 3	Colony 4
Gram staining	Gram +ve Rod	Gram +ve Rod	Gram +ve Rod	Gram +ve Rod
Endospore staining	Positive (+ve)	Positive (+ve)	Positive (+ve)	Positive (+ve)
Starch hydrolysis	Positive (+ve)	Positive (+ve)	Positive (+ve)	Positive (+ve)
Motility test	*NA	*NA	Positive (+ve)	*NA
VP test	Positive (+ve)	Positive (+ve)	Positive (+ve)	Positive (+ve)
Citrate utilization test	Positive (+ve)	Positive (+ve)	*NA	*NA
Mannitol fermentation	*NA	*NA	*NA	Positive (+ve)
Growth at 55^0 c	Negative (- ve)	Positive (+ve)	*NA	*NA
Identified Bacteria	***Bacillus subtilis***	***Bacillus licheniformis***	***Bacillus cereus***	***Bacillus polymyxa***

NA – procedure/biochemical test not adapted/not done.

All the four bacteria were screened for Bacitracin production and only *Bacillus subtilis* showed positive results for production.

3.2 Antibacterial activity of Bacitracin

The antibacterial activity for crude was first screened against *Staphylococcus aureus* (Figure 1a & 1b) and *Micrococcus luteus* (Figure 1c). (Table 2)

Table 2: Antibacterial activity of crude extract of Bacitracin showing a zone of inhibition.

Test organisms	***Staphylococcus aureus***				***Micrococcus luteus***			
Concentration	**70 µl**	**80 µl**	**90 µl**	**100 µl**	**70 µl**	**80 µl**	**90 µl**	**100 µl**
Zone of inhibition	18mm	18mm	22mm	22mm	20mm	20mm	26mm	26mm

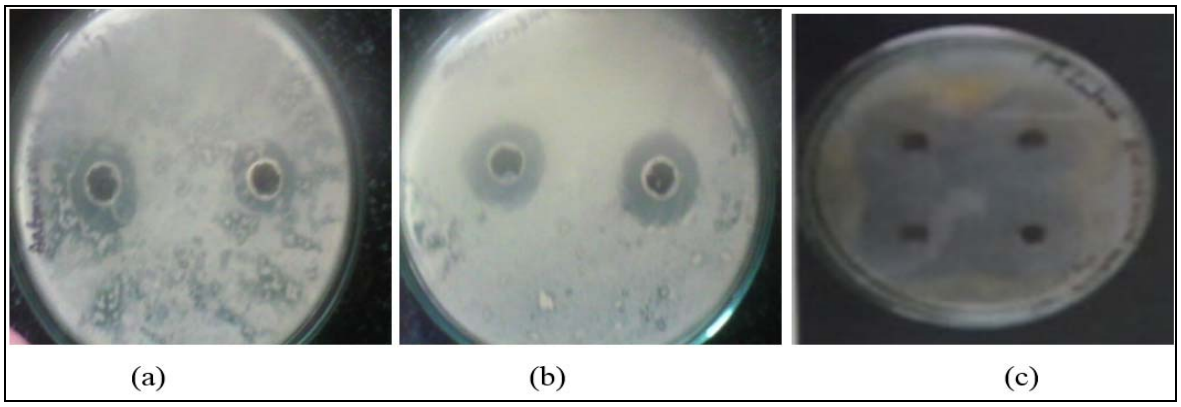

Fig 1: Screening of crude bacitracin against *Staphylococcus aureus* at (a) 70 µL and 80 µL (b) 90 µL and 100 µL and against *Micrococcus luteus* (c) at different volumes.

Extracted and purified samples were subjected to agar well diffusion method and zone of inhibition was measured in mm. More zone of inhibition was measured in purified samples as compared to crude extracts (Table 3) (figure 2).

Table 3: Antibacterial activity of purified Bacitracin zone of inhibition

Test organisms	**Zone of inhibition**
Staphylococcus aureus	24mm
Micrococcus luteus	27mm

(a) (b)

Fig 2: Zone of inhibition for purified extract of Bacitracin against (a) *Staphylococcus aureus* (b) *Micrococcus luteus.*

Purified Bacitracin was identified by thin layer chromatography and its R_f value was found to be 0.95 close to the retardation factor of the standard bacitracin (i.e. 0.96). (Table 4)

Table 4: Results of TLC identification of Bacitracin

Test organism	Solvent font	Solute font	R_f value
Standard bacitracin	7.3 cm	7.0 cm	0.96 cm
Sample bacitracin	6.4 cm	6.1 cm	0.95 cm

3.3 Optimization of media

The optimal condition for bacitracin production was carried out for carbon sources, nitrogen sources, pH of the production media, incubation temperature and incubation time. The better conditions were analysed as the bactericidal effect for each conditions against *Staphylococcus aureus* and *Micrococcus luteus*. The larger zone of inhibitions against these standard pathogenic microbes indicates the better potency and efficacy for bacitracin production. (Table 5) (Figure 3-7).

Table 5: optimization for different conditions for better bacitracin production.

Optimization of conditions	Zone of inhibition (in mm)	
	Staphylococcus aureus	***Micrococcus luteus***
Optimization by Carbon Source (figure 5)		
Glucose as Carbon Source	15 mm	14 mm
Sucrose as Carbon Source	09 mm	10 mm
Lactose as Carbon Source	10 mm	11 mm
Fructose as Carbon Source	12 mm	08 mm
Optimization by Nitrogen Source (figure 6)		
Aspargine as nitrogen source	12 mm	11 mm
Methionine as nitrogen source	05 mm	06 mm
Yeast Extract as nitrogen source	01 mm	00 mm
Peptone as nitrogen source	04 mm	02 mm
Sodium nitrite as nitrogen source	02 mm	01 mm
Ammonium nitrite as nitrogen source	02 mm	01 mm
Optimization by pH (figure 7)		
pH at 5	10 mm	15 mm
pH at 6	15 mm	20 mm
pH at 7	30 mm	35 mm
pH at 8	10 mm	20 mm
pH at 9	20 mm	25 mm
Optimization by incubation temperature (ºC) (figure 8)		
Incubation temperature at -4(ºC)	10 mm	10 mm
Incubation temperature at 37(ºC)	40 mm	50 mm
Incubation temperature at 42(ºC)	45 mm	60 mm
Incubation temperature (ºC) at room temp.	20 mm	10 mm
Optimization by incubation hours (in hours) (figure 9)		
Incubation hours at 48 hours	00 mm	00 mm
Incubation hours at 72 hours	10 mm	10 mm
Incubation hours at 96 hours	10 mm	15mm
Incubation hours at 120 hours	20 mm	30 mm
Incubation hours at 144 hours	55 mm	60 mm

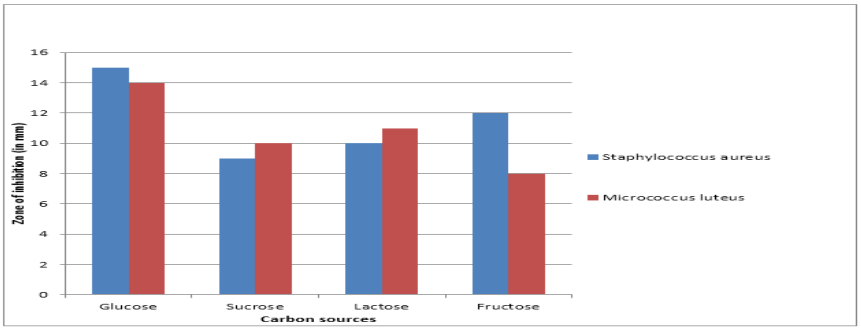

Fig 3: Optimization of carbon source.

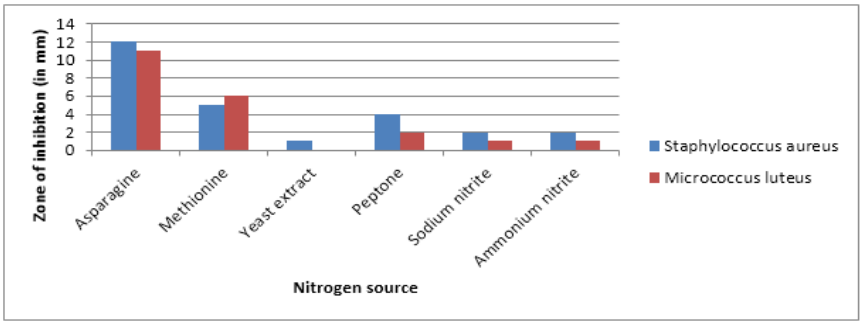

Fig 4: Optimization of nitrogen source.

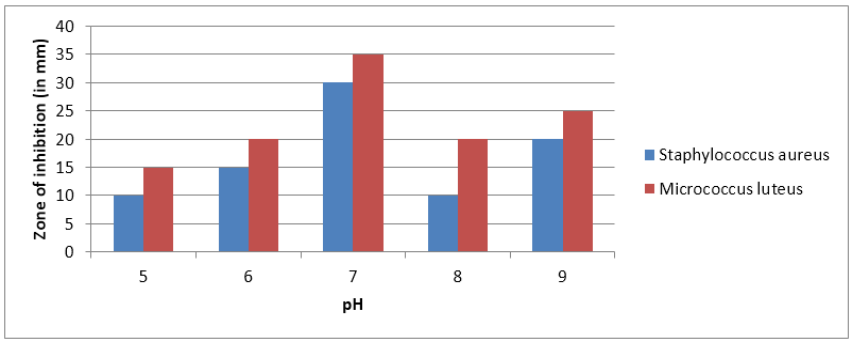

Fig 5: Optimization of pH

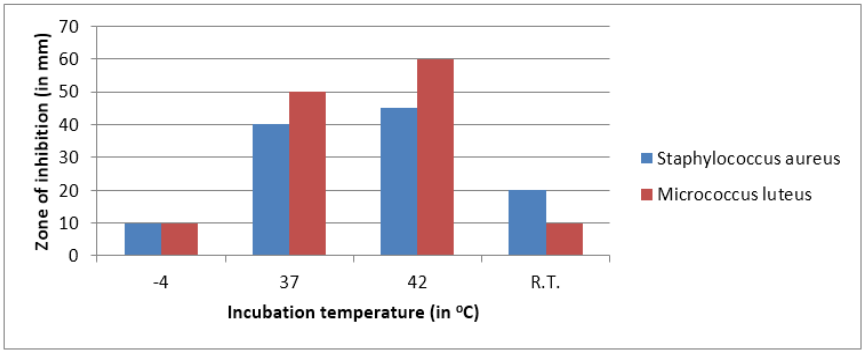

Fig 6: Optimization of temperature (in °C).

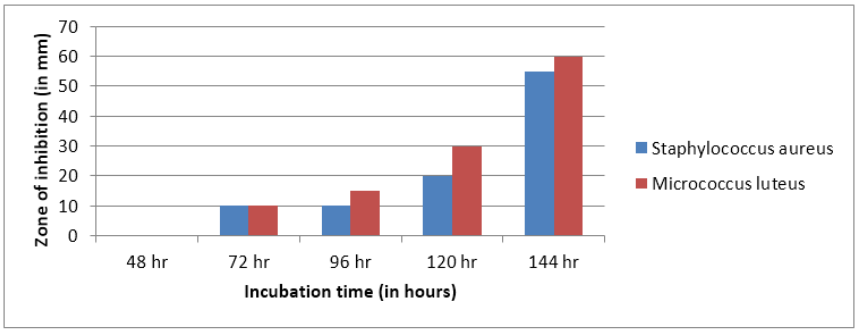

Fig 7: Optimization of incubation time (in hours).

4. Conclusion

Bacillus subtilis was isolated and screened for the bacitracin production. Bacitracin was produced by using specific production, media and characterized by their antimicrobial activity against the test pathogenic organisms such as *Staphylococcus aureus* and *Micrococcus luteus*. Bacitracin was purified by Butanol-Ether solvent system. The purified bacitracin was characterized by comparing the R_f value in thin layer chromatography with the standard bacitracin commercially available. The R_f value of purified one was 0.95 that was closer to the R_f value of the standard bacitracin of 0.96. The production, media was optimized by changing the culture conditions such as carbon source, nitrogen source, pH, temperature and incubation period and analyzing against the antimicrobial activity against *Staphylococcus aureus* and *Micrococcus luteus*. The best result were at 144 hrs of incubation by using glucose as carbon source, asparagine as nitrogen source with the pH of media at 7 and incubation period of 42 ^{0}C.

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