

“ISOLATION OF ANTIBIOTIC PRODUCING *ACTINOMYCETES* FROM SOIL”

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ABSTRACT

The present study deals with the isolation, production, purification and optimization of antibiotics from soil. The soil samples were collected from various places. *Actinomycetes* strains were isolated in specific medium using starch casein agar medium. The *actinomycetes* were screened with regard to potential against gram positive and gram negative bacteria. The purified *actinomycetes* strains were performed in biochemical tests. Gram staining, sugars fermentation, HS₂ production, urease production, MR-VP test, indole production, citrate utilization. Fermentation was carried out with starch casein broth and identified strain of *actinomyces* as inoculums. Antimicrobial activity of antibiotics was detected on Mueller Hinton Agar Media against E.coli. The purification of antibiotics was done with the help of activated charcoal. During the experiment a modified fermentation medium was formulated and activity of antibiotic produced in this medium was detected against E.coli. The isolated bacteria such as *actinomycetes* obtained from the soil.

INTRODUCTION

Antibiotics are complex chemical secondary metabolites, which are produced by microorganisms & act against other microorganisms. The term antibiotics had its origin from Greek word anti-against+biotikos =”fit for life”. Antibiotics are the best known products of *actinomycetes*. The best known genus of *actinomycetes*; Streptomyces (Order Actinomycetales, family Streptomycetaceae are gram-positive, filamentous bacteria that are ubiquitous in soil and produce the majority (>70%) of known antibiotics (Tanaka and Omura, 1990). The isolation & characterization of *Actinomycetes* were performed in different biochemical methods. Morphological examination of the *Actinomycetes* was done by using cellophane tape & cover slip buried methods. The mycelium structure color & arrangement of conidiophores & arthroscopy on the mycelium were examined under oil immersion (100X). Different biochemical tests were performed to characterize the *Actinomycetes*. The tests generally used are starch hydrolysis, Triple sugar Iron (TSI) agar test, citrate utilization test, indole test, methyl red test, voges-proskauer (acetone production) test, catalase test. The present study is an attempt to produce antibiotics from *actinomycetes*, isolated from soil, by fermentation & the determination of their antimicrobial activity.

Production of Antibiotic:-Starch casein broth medium was used as suitable medium for the production. Prepared starch casein broth medium by adding starch, peptone beef extract, casein (1% sol.) in distilled water, casein solution pasteurized at 72⁰c for 30min. When medium is prepared then inoculate the *actinomycetes* into the broth. Then fermentation broth incubated in the shaker incubator for 7-8days at 37⁰c.

MATERIAL & METHODS

Methods:- The activity of Produced antibiotic was demonstrated by Kirby baurer method. For this test Muller Hinton Agar medium was used. A clear zone was shown in the Mueller Hinton Agar plate against E.coli after 4-5 days of incubation of fermented starch casein agar medium and a clear zone was observed against E.coli after 24-30 hours of incubation of fermented modified medium.

1) Collection of Soil samples:-

All the soil samples were collected from various places, near drainage, mud & coloured soil where there were no plants around. Soil samples collected the underlying surface soil of about 10cm. 1g of soil was taken in a sterilized flask & to this 100ml of sterile water for serial dilution.

2) Screening of soil samples by crowded plate technique:-

A series of culture tubes containing 9ml of sterile water were taken. From the stock culture, 1ml suspension was transferred aseptically to the 1st tube (10^{-1}), mixed well. From the 1st tube, 1ml of suspension was transferred into the 2nd tube (10^{-2}), mixed well. Similarly, dilution (10^{-1} to 10^{-5}), 0.1ml of suspension was spread on starch casein agar medium plates. The plates were incubated at 28^oc for 5 days. The plates were observed daily during incubation. After 5 days, pinpoint colonies which were characteristics of *actinomycetes* with inhibitory/clear zone around it, were picked & purified into new *Actinomycetes* isolation agar plates. The strains were purified by multiple streaking methods on *actinomycetes* agar medium. A stock culture of each strain was maintained in an *Actinomycetes* agar slants & kept in refrigerator for further use

Characterization of actinomycetes:-

1) Morphological characteristics:-

Morphological & cultural characters of strain were studied by inoculating strain into sterile media like, yeast – malt extract agar. The media were sterilized & poured into sterile petri dishes. After solidification of the media, culture of the selected strain was streaked & incubated at 27^oc for 7 days.

2) Microscopical characterization:-

a) Gram staining method:-A smear of the strain was prepared on a clean glass slide & the smear was allowed to air dry & then heat fixed. The heat fixed smear was flooded with crystal violet & after one minute, it was washed with water & flooded with mordant Gram's iodine. The smear was decolorized with 95% ethyl alcohol, washed with water & then counter – stained with safranin for 45sec. After washing with water, the smear was dried with tissue paper & examined under oil immersion (100X).

b) Motility:-The motility is an important part in identification of bacteria. Motility of the given isolates, was observed by using 'Hanging drop Method' as per standard protocol. On the basis of motility the organisms were observed for their motility as no-motile, sluggishly motile,

actively motile or highly motile. The bacterial isolates were further followed for biochemical characteristics.

3) Biochemical characteristics:-

1) **Sugar fermentation test:-** The bacterial isolates were tested for their ability to utilize a variety of sugars such as glucose, lactose, sucrose and mannitol as a source of carbon and energy to be tested for production of gas. The sugar to be tested was added in peptone water medium to the concentration of 1% and autoclaved. The isolate was inoculated and incubated in the medium at 37⁰c for 24 hours.

2) **Indole test:-** This test is performed to determine the ability of an organism to split indole from tryptophan molecules. Certain organisms are capable of hydrolyzing and deaminating the amino acid tryptophan that mediates the production of indole. Tryptone water a medium for detection of indole production which is rich in tryptophan was inoculated with the bacterial isolate and incubated at 37⁰c for 24 hours. At the end a few drops of Kovac's reagent was added and the tube was observed for formation of a red coloured ring. The presence of a red coloured ring gives a positive test while absence of red coloration gives a negative test.

2) **Methyl red test:-** This test provides an important key point for the identification of bacteria that produce strong acid from glucose as well as maintain low pH after prolonged incubation by the buffering system. Two drops of methyl red reagent were added after 24 hours of incubation at 37⁰c. The change of colour of medium to bright red colour after the addition of methyl red indicator. Shows a positive test while no change in colour shows a negative test.

3) **Voges-Proskauer test:-** This test is used to detect the production of acetyl methyl carbinol. The tube containing glucose phosphate broth was inoculated with bacterial isolate and incubated at 37⁰c for 24 hours. After incubation about 0.6 ml of alpha naphthol solution followed by 0.2 ml of 40% KOH solution was added to 2.5 ml of incubated broth. The tubes were shaken well and kept for 10-15 min. The positive test is indicated by development of red colour after the addition of Barritt's reagent. The red colour is due to production of acetyl methyl carbinol. The formation of red colour gives a positive test while no change in colour gives a negative test.

4) **Citrate utilization test:-** This test is used to determine the ability of an organism to use sodium citrate as a sole source of carbon for metabolism & growth. Koser's citrate broth which is devoid of protein and carbohydrate. Except citrate was inoculated with organisms and incubated at 37⁰c for 24 hours. The broth used for the test was Koser's citrate broth which contains bromothymol blue indicator which is yellow at acidic pH, green at neutral pH and blue at alkaline pH. The presence of blue colour in the broth indicates a positive test while absence of blue colour indicates a negative test.

5) **Urease test:-** This test is used to determine the production of enzyme urease by micro-organism. For this test urea agar base is used. It contains urea which gets hydrolyzed by the growing bacteria. Hydrolysis of urea results in the formation of ammonia, which is identified by the change in colour from yellow to pink because of the indicator incorporated in the medium i.e phenol red. The urea agar base slant was inoculated with the bacteria isolate and was incubated at 37⁰c for 24 hours. After 24 hours the formation of pink colour indicated a positive test while no change in colour indicated a negative test.

6) **Triple sugar iron agar:-** Triple sugar iron agar is used for the differentiation of enteric pathogens by ability to determine carbohydrate fermentation and hydrogen sulphide production. Organisms that ferment glucose produce a variety of acids turning the colour of the medium from red to yellow, more amounts of acids are liberated in butt (fermentation) than in the slant (respiration). Growing bacteria also form alkaline products from the oxidative decarboxylation of peptone and these alkaline products neutralize the large amount of acid present in the butt. Thus

the appearance of an alkaline (red) slant and an acid (yellow), but after incubation indicates that the organism is a glucose fermenter but is unable to ferment lactose and for sucrose. Bacteria that ferment lactose or sucrose (or both) in addition to glucose produce large amounts of acid, enabling no reversion of pH in that region and thus bacteria exhibit an acid slant and acid butt. Gas production (co) is detected by the presence of cracks or bubbles in the medium, when the

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accumulated gas escapes. This sulphate is reduced to hydrogen sulphide by several species of bacteria and HS combine with ferric ions of ferric salt to produce the insoluble black precipitate

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of ferrous sulphide. Reduction of thiosulphate proceeds only in an acid environment and blackening usually occurs in the butt of the tube.

Alkaline slant/acid butt = only glucose fermented.

Acid slant/acid butt = glucose and sucrose fermented or glucose and lactose fermented or all the three sugars fermented.

Bubbles or cracks present = gas production.

Black precipitate present = HS gas production..

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Antimicrobial activity test of fermentation broth:-The activity of produced antibiotic was demonstrated by antimicrobial activity test, for this test Muller Hinton agar medium was used. Antimicrobial activity test performed for confirmation of antibiotic production. Steps involved in the test are: Prepared Muller Hinton agar medium & sterilized, poured it into sterile Petri plates & allowed to solidify, made a uniform layer of E.coli culture by using L-rod, and made a well in the centre of the agar plate. Place 50 microliter of supernatant of culture broth in the well, Incubate the plate in the incubator for 24 – 48 hrs at 37⁰c. Activity of antibiotics is demonstrated on the basis of zone size. Larger zone shows maximum production of antibiotics.

Purification of the antibiotic:-A general laboratory method was used for the purification of antibiotics. In this method centrifuged the fermentation media for 10min. at 6000rpm 7 4⁰c, Collect supernatant & filtered by using wattmann's no.1 filter paper, Taken filtrate, added 2 present (2g in 100ml) activated charcoal kept the solution in the shaker incubator for 20min. , Filter the solution by using wattmann's no. 1 filter paper, after 2min.added 10ml of phosphate buffer in the residue,Takenfiltrate & allowed it for crystallization at room temperature, after crystallization added 2-3ml of phosphate buffer for dissolving the crystal.

Optimization on the basis of production medium composition:-

Inoculated the actinomycetes in each broth. Incubated the fermentation broth in the shaker incubator at 37⁰c for 3-4 days and then activity of streptomycin produced in the modified medium was determined by an antimicrobial activity test.

RESULT & DISCUSSION

The isolated bacteria were obtained from soil samples. The morphological characters are still widely used for characterizing genera. Gram positive bacteria were observed in microscopic study of gram staining slides with purple colour, rod shape and filamentous structure. Isolates were tested by biochemical tests for the identification of bacteria.

Biochemical characteristics of isolated gram positive rod shaped

bacteria 1) Sugars test:-

Test	Observation	Result
Glucose	Acid & Gas Production	Positive
Lactose	Acid Production	Positive
Mannitol	Acid Production	Positive
Sucrose	Acid & Gas Production	Positive

From the above observation it was found that the acid & gas production in glucose & sucrose test and acid production by test microorganism in lactose & mannitol test.

IMViC Test:-

Test	Observation	Result
Indole	Cherry red colour ring	Positive
MR	Red colour	Positive
VP	No Red colour	Negative
Citrate	Blue colour	Positive
Urease	Pink Colour	Positive
H ₂ S Production(TSI Slant)	No H ₂ S Production	Negative

From the above observation it was found that the cherry red ring formed in the tube inoculated with test microorganisms for indole test. The red colour formed in the tube inoculated with test microorganisms for methyl red test. The no colour change in the tube inoculated with test microorganisms for voges proskauer test. The blue colour formed in the tube inoculated with test microorganisms for citrate utilization test. The colour change that is pink colour observed in the tube containing urea agar slant for urease test. This indicates the production of citrase enzyme by micro-organism. The no colour change in the tube inoculated with micro-organism indicated that no production of HS in the TSI slants.

From above results, the isolated bacteria were identified as *Actinomyces*. Whitish colonies of the *Actinomyces* were observed on the surface of the starch casein agar medium. The *Actinomyces* were purified by multiple streaking methods on *Actinomyces* agar medium.

CONCLUSION

Antibiotics, because of their industrial importance, are the best known products of *actinomyces*. The primary objective was to isolate the *actinomyces* which have capacity to produce new antibiotics and characterize it. The taxonomical studies which confirmed the typical biochemical behaviours resembling *actinomyces*. These tests helped to design the fermentation medium for antibiotic production. The strain was selected based on its significant broad spectrum antibacterial activity against both gram positive and gram negative bacteria. This strain was further studied for its morphological, microscopically and cultural characteristics for classification of its taxonomic identification. From morphological and taxonomic studies, it was observed that the strain was considered to belong to the Genus: *Actinomyces*.

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