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Research Article

PRODUCTION AND CHARACTERIZATION OF ANTIBIOTICS FROM SOIL-ISOLATED ACTINOMYCETES

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ABSTRACT
In the present study, the soil samples were collected from various places in the Coimbatore, near drainage, mud and coloured soil where there were no plants around. Actinomycetes strains were isolated in specific medium using soyabean casein digest (SBCD) medium. These Actinomycetes were screened with regard to potential against Gram-positive and Gram-negative bacteria. The purified Actinomycetes strains were performed in biochemical tests. Crowded plate technique was used for the isolation of actinomycetes in media such as soybean – casein digest medium and actinomycetes isolation agar. In a medium formulation study, A-5 and A-5 actinomycete mutant strains were evaluated for maximum antibiotic production using various carbon and nitrogen sources. Zone of inhibition and packed cell volume were the parameters used for the evaluation. Various fermentation conditions such as pH, temperature and dissolved oxygen were also optimized for maximal production of antibiotic from both A-5 and A-5 mutant. Findings from this investigation reveal that strain A-5, in that order, exhibited superior antimicrobial activities to other soil isolates of actinomycetes.
KEYWORDS: Actinomycete, Antibiotic, MIC, Fermentation.

INTRODUCTION

Scientists are working hard to find new ways to defeat bacteria that are increasingly resisting the antibiotics already available. These range from continuing to develop new antibiotics to keep up with bacteria's rapid evolution to giving bacteria themselves disease - bugs are, like larger organisms, susceptible to viruses. Since the introduction of penicillin bacteria have been proving themselves ever more adept at defeating antibiotics - soon after its introduction in the 1940s resistant strains of bacteria began to appear[1]. Microorganisms constitute an inexhaustible reservoir of compounds with pharmacological, physiological, medical or agricultural applications[2-4]. Antibiotics continue to play a crucial role in the development of tissue culture techniques and basic screenings, primarily in biochemistry, molecular biology, microbiology and genetics (including genetic engineering) and to a lesser extent, pharmacology and organic chemistry. Actinomycetes are the most fruitful source for production of bioactive secondary metabolites. Actinomycetes a total 7899 (100%) compounds has been identified up to 1988; 67% from Actinomycetes, 12% from bacteria and 20% fungi. Japanese and American scientists[5] contributed greatly to the discovery of Actinomycetes products, 286 compounds are produced by Streptomyces hygroscopicus, 189 by S. griseus, 129 by S. lavendulae, 383 by Micromonospora app., 278 by Nocardia sp. The isolation and characterization of Actinomycetes were performed in different biochemical methods[6]. Morphological examination of the Actinomycetes was done by using cellophane tape and cover slip-buried methods[7]. The mycelium structure, color and arrangement of conidiophores and arthrospore on the mycelium were examined under oil immersion (1000X). The observed structure compared with Bergay's manual of Determinative Bacteriology, Ninth edition (2000) for identification Streptomyces sp., under the group of Actinomycetes. 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, vogus-proskauer (Acetone Production) test, Catalase test. The Actinomycetes were originally considered an intermediate group between bacteria and fungi then were recognized as prokaryotic organisms. Actinomycetes species synthesize a numerous natural metabolites with diverse biological activity such as antibiotics. Antibiotics of Actinomycetes origin evidence a wide variety of chemical structure, including amino glycosides, anthracyclines, β-lactams, nucleosides,

peptides, polyenes, actinomycins and tetracycline[8]. Generally, antibiotics are non-growing associated secondary metabolites.

The present study is an attempt to produce antibiotics from actinomycetes, isolated from soil, by fermentation and the determination of their antimicrobial activity.

MATERIALS AND METHODS

Materials

Nutrient agar media , dextrose agar media, Yeast extract pest ,Tyrosine – S.D. Agar-agar bacteriological, Potassium nitrate ,Peptone ,Beef extract – Qulaigenes ,Starch agar media –Ferric ammonium sulphate, Dibasic potassium phosphate, Sodium thiosulphate, Calcium carbonate – S.D. fine chemical Ltd., Mumbai, Acetic acid, Ammonium sulphate, Ammonium chloride, ISP Media no. 3, Nutrient broth media, Muller hinton agar media, Muller hinton broth media, Ethyl acetate, Ferrous sulphate, Zinc sulphate, $Mncl_2.4H20$, Malt extract, Glycerol, Iodine solution, Sulphanilic acid, Ethyl alcohol.

Isolation of actinomycetes from soil

Collection of Soil samples

All the soil samples were collected from various places in the Coimbatore, near drainage, mud and coloured soil where there were no plants around. Soil samples were collected the underlying surface soil of about 10cm. 1g of soil was taken in a sterilized flask and to this 100ml of sterile water for serial dilution[9, 10].

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 2nd tube. (10^{-2}), mixed well. Similarly, dilutions up to 10^{-5} were made. From each dilution (10^{-1} to 10^{-5}), 0.1ml of suspension was spread on soyabean casein digest (SBCD) medium plates. The plates were incubated at 28°C for 5days. The plates were observed daily during incubation. After 5 days, pinpoint colonies which were characteristic of actinomycetes with inhibitory/ clear zone around it, were picked and purified into a new Actinomyces isolation agar plates. Total of 13 colonies were selected for the study which was named as A_1, A_2, and A_3 …. A_{13}. The strains were purified by multiple streaking methods on actinomyces agar medium. A stock culture of each strain was maintained in an Actinomyces agar slants and kept in refrigerator for further use[11, 12].

Test for microbial sensitivity

The microbial sensitivity of the isolates was analyzed by agar streak method. Each of the isolate was streaked as a straight line on soyabean casein digest (SBCD) medium. Petri dishes separately and incubated at 27°C for about 6 days. After 6 days, different strains of bacteria were streaked at right angle to the streak and incubated at 37°C for 24 hours.

Characterization of actinomycetes

Taxonomical Characterization

Soil isolates were classified into various genus by studying its biochemical characters[13].

Test for Melanoids pigmentation formation

Pigment production is one of the most significant properties of Actinomycetes. Most of the soil Actinomycetes produces melanin pigment in Waksman medium. The isolates were streaked onto sterile Waksman medium and incubated for 4days at 37°C.

Test for Proteolytic activity

Milk coagulation and peptonisation

Casein is a primary protein in skim milk. Actinomycetes usually digest milk protein. The protein which is present in skim milk, if get digested giving positive reaction. To study the Proteolytic activity, soil isolates were inoculated in different culture tubes containing sterile pasteurized skimmed milk and incubated at 35°C for 48 hours. The tubes were observed daily for 48 hours. Reduction of litmus paper, Change in medium colour. These changes take place due to the digestion of milk proteins and change in the pH of the medium.

Gelatin Liquefaction test

This test is done on sterile nutrient gelatin media. Gelatin is a protein that digested by Actinomycetes extracellular enzymes called gelatinase. Nutrient gelatin contains beef extract and peptone to support growth and enough gelatin (120g/l) to cause medium gel. Isolates were inoculated into a nutrient gelatin tubes with a sterile needle. The tubes were incubated at 35°C. The medium chilled thoroughly into the refrigerator before examination. Chilling is essential because gelatin is liquid at temperature above 20°C. Actinomycetes can hydrolyze gelatin by its exoenzyme. This can be studied by inoculating isolates on to the sterile nutrient gelatin medium and incubated for 10 days at room temperature.

Test for Amylolytic activity

Amylolytic activity of soil isolates were studied by using starch agar medium. Starch is a polysaccharide composed of repeating alpha-D-glucose subunit. Actinomycetes produce enzyme amylase which breakdown starch into single subunits of alpha-D-glucose. This medium contains beef extract and peptone to support growth, soluble starch and agar. For this test, the cultures were grown for a period of 5 to 7 days on starch agar medium at 28°C. Amylolytic activity was

observed by using iodine solution which indicates the hydrolysis of starch.

Carbohydrate Assimilation test

Type of carbohydrate source utilized by Actinomycetes, is an important biochemical property for identification of Actinomycetes. Type of carbon source utilized by micro organisms was identified by growth around or under discs along with or without colour change of the carbohydrate utilization agar medium (ISP No.9). One ml of soil isolate inoculated into the sterile carbohydrates utilization agar (ISP MEDIUM No. 9) with bromocresol purple dye. After solidification sterile discs containing 3% dextrose, starch, lactose, maltose, were placed aseptically onto the surface and incubated at 27°C for 5-10 days. The presence of growth around or under the discs along with or without change in colour of the medium was observed.

Acid production

Soil isolates were inoculated in sterile glucose nutrient broth and incubated at 28°C for 15 days. The colour change in the broth from blue to yellow was observed.

Hydrogen sulfide production

Numerous Actinomycetes are able to ferment the proteins and produce hydrogen sulphide. The sterile Petri dishes of H_2S production medium were streaked with soil isolates and incubated at 37°C for 4 days. After incubation period, H_2S production was observed by rotten egg smell and change in colour of the medium to greenish brown, bluish black or black colour.

Morphological Characterization

Cultural characterization

Morphological and cultural characters of strain were studied by inoculating the strain A_5 into sterile media like, Yeast-malt extract agar (ISP- 2), Oatmeal agar (ISP3), Inorganic Salts -Starch Agar (ISP-4). All the media were sterilized and poured into sterile Petri dishes. After solidification of the media, culture of the selected strain was streaked and incubated at 27°C for 7 days.

Microscopical characterization

Gram staining method

A smear of the selected strain (A-4) was prepared on a clean glass slide and the smear was allowed to air-dry and then heat fixed. The heat-fixed smear was flooded with crystal violet and after one minute, it was washed with water and flooded with mordant Gram's iodine. The smear was decolorized with 95 % ethyl alcohol, washed with water and then counter-stained with safranin for 45 s. After washing with water, the smear was dried with tissue paper and examined under oil immersion (100 x).

RESULTS AND DISCUSSION

Characterization of soil isolates

Screening of Soil

The soil isolates of Actinomycetes having pin point colonies with zone of inhibition cultured by Crowded Plate technique. Isolations were carried out by multiple streak method on Actinomyces agar media to obtain pure culture of 13 strains. Each strain was preserved in an Actinomyces agar slants. Results are shown in figure. 1.

Test for Microbial Sensitivity

The isolated strains were tested for microbial sensitivity using different strains of bacteria by Agar Streak method. Out of the 13 actinomycetes screened six strains namely A_2, A_4, A_5, A_9, A_{12}, and A_{13} showed significant antimicrobial activity against both Gram positive and Gram negative organisms used in the test. However A_5 showed a very broad spectrum with higher scores than all other strains; Results are shown in (figure. 2). The six actinomycetes strains which were selected through the sensitivity test were further taken for taxonomical characterization.

Taxonomical characterization

In the taxonomical characterization, melanoids formation (figure. 3), milk coagulation and peptonization, gelatin liquefaction, acid productions are shown in (figure 4), H_2S production (figure. 5), starch hydrolysis(figure. 6), carbohydrates assimilation test (figure. 7) were carried out. Results are shown in Table. 1. All six strains namely A_2, A_4, A_5, A_9, A_{12} and A_{13}, were giving positive result in starch hydrolysis test. Strain A_2 and A_5 gave positive results in pigment production tests and A_4, A_9, A_{12} and A_{13} gave negative results in melaniod formation but it produced growth in above said test media. All the strains were giving positive results in test for Proteolytic activity except A_2 and A_{13}. All the strains except A_2 and A_{13} are able to produce the acid in the Acid production test. These physico-chemical properties helped to differentiate the genus of actinomycetes. Therefore, based on the above results A_4, A_5, A_9, A_{12} were grouped belong to the genus Actinomyces and the strains A_2, A_{13} belong to the genus Nocardia. Among the 13 isolates tested, strain (A_5) showing prominent antimicrobial activity and so took strain (A_5) for further detailed investigation regarding the optimization and large scale bio-processing of its antibiotic production. In the taxonomical characterization, carbohydrate assimilation test was carried out.

Morphological and Cultural Characterization

The Morphological and cultural characteristics of the organisms were studied on International Streptomyces Project (ISP) media. The different Mediums were used

for study like ISP- 2, ISP- 3 and ISP- 4. Results are observed and summarized in Table 2.

CONCLUSION

Most of the Microorganism has developed resistance to the existing antibiotics so it has provoked the need of a constant research on production of newer antibiotics in order to overcome the resistant microorganism. Antibiotics, because of their industrial importance, are the best known products of actinomycetes. The primary objective was to isolate the actinomycetes which having capacity to produce new antibiotic and characterize it. Soil sample collected from different place than tested their microbial sensitivity against different strains of bacteria. Initially 13 strains of microorganism were isolated. Out of 13 strains only 6 were selected based on their antibacterial activity. After than did taxonomical studies which confirmed the typical biochemical behaviors resembling actinomycetes. These tests helped to design the fermentation medium for antibiotic production. The strain A_5 was selected based on its significant broad spectrum antibacterial activity against both Gram positive and Gram negative bacteria. This strain was further studied for it's morphological, microscopically and cultural characteristics for classification of its taxonomical identification. From morphological and taxonomical studies, it was observed that the strain A_5 was considered to belong to the Genus: Actinomyces.

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REFERENCES

1. Hoopwood DA. Antibiotics: Opportunities for genetic manipulation, JSTOR Philosophical Transaction of the royal society of London. series B, Biological., 1989; 324(1224): 1-3.
2. Smith JE. Perspective in biotechnology and applied microbiology. Murray Moo-Young. 1989; 105-134.
3. Bulock J. Basic Biotechnology. Academic Press, 1987; 425-448.
4. Vandemme EJ. Biotechnology of Industrial Antibiotics. Dekker Series, Marcel Dekker Inc., New York, 1948; 22: 3-42.
5. Tanaka Y, Omura S. Metabolism and products of actinomycetes-an introduction.Actinomycetologica. 1990; 4: 13-14.
6. Dhanasekaran D, Selvamani S, Panneerselvam A, Thajuddin N. Isolation and characterization of actinomycetes in vellar estuay, annagkoil, tamilnadu. Afr.J.Biotechnol., 2009; 8: 4159-4162.
7. Williams ST, Cross T. 1971. Actinomycetes. In: Methods in Microbiology, Booth. (Eds.). Academic press, London.
8. Barrios-Gonzalez J, Fernandez FJ, Tomasini A, Megia A. Secondary metabolites production by solid-state fermentation. Malaysian J. Microbiol., 2005; 1: 1-6.
9. Sharma DD. Distribution of Antifungal Actinomycetes in soils. Indian J. Microbiology. 1989; 29(2): 169-171.
10. Augustine SK, Bhavasar SP, Kapadnis BP. Production of a growth dependent metabolite active against dermatophyates by Streptomyces rochei AK 39. Indian J. Med. Res., 2005; 121: 164-170.
11. Abbas Ibrahim H. A Biological and Biochemical Studies of Actinomycetes Isolated from Kuwait Saline Soil-Kuwait. Journal of applied science research. 2006; 2(10): 809-815.
12. Okudoh V, Wallis FM. Antimicrobial activity of rare Actinomycetes isolated from natural habitats in Kwazulu-Natal. South African journal of science. 2007; 103: 216- 222.
13. Antonova-Nikolova Stefka, Tzekova Nikoleta, Yocheva Ljubomira. Taxonomy of Streptomyces SP. Strain 3B. Bio line International journal of culture collections. 2004; 4(1): 36-42.

Table 1. Taxonomical characterization

Soil Isolates	Melanoid formation	Milk co-gulation and Peptonization	Gelatin liquefaction	Starch hydrolysis	Carbohydrate assimilation	Acid Production	H_2S Production.
A_2	Light brown pigmentation	-	-	+	Glucose	-	-
A_4	Only growth	Clear with acidic reaction	+	+	Glucose	+	-
A_5	Light brown pigmentation	clear with slightly acidic reaction	+	+	Lactose Starch	+	-
A_9	Only growth	Clear with acidic reaction	+	+	Starch	+	+
A_{12}	Only growth	Not clear with acidic reaction	+	+	Lactose	+	-
A_{13}	Only growth	-	-	+	Lactose	-	-

Table 2. Morphological and Cultural Characterization

Medium Used	A_5
ISP – 2	Even circular, rough(matte like), elevation, opaque, graying pigmentation, pinpoint, unduate
ISP – 3	Pinpoint, circular droplet, convex, black pigmentation.
ISP – 4	Unduated, dull rough, entire, convex, irregular, viscous.



Figure 1. Microbial Colonies by Crowded Plate Technique

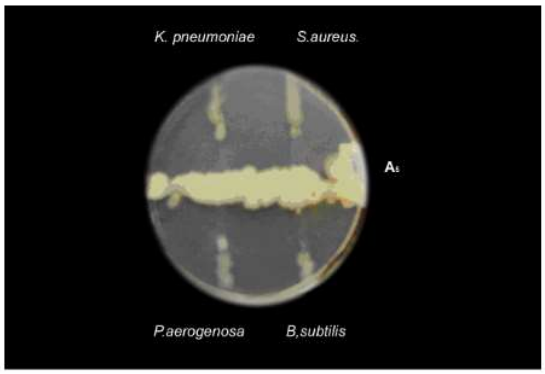


Figure 2. Sensitivity of Microorganism towards StrainA_5

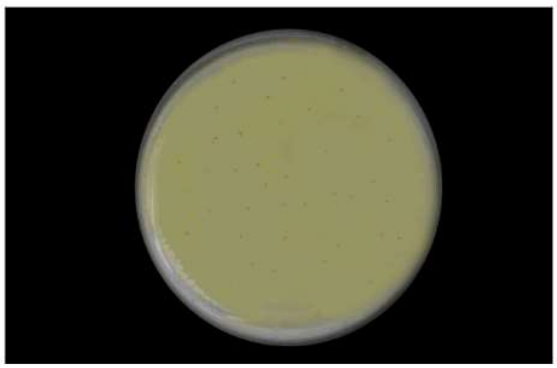


Figure 3. Melanoid formation test of StrainA_5

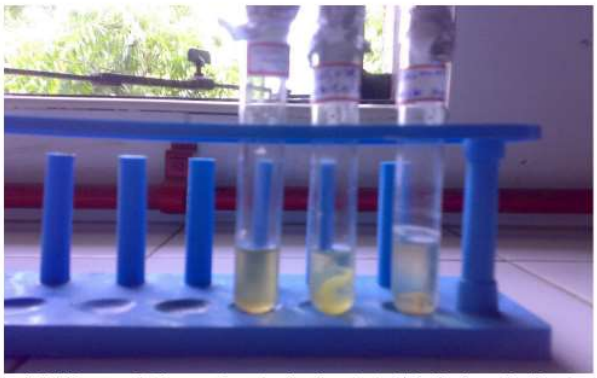


Figure 4. Milk coagulation and peptonisation test, Gelatin liquefaction test, and Acid production test of StrainA_5

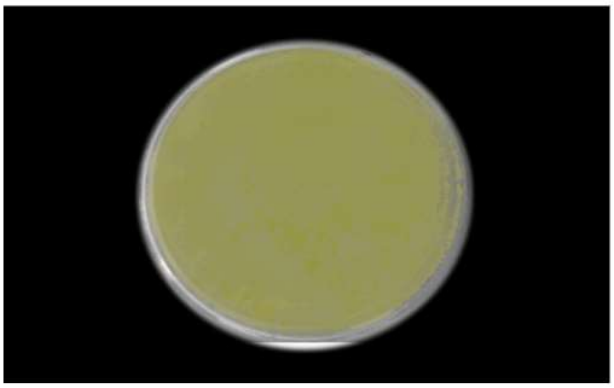


Figure 5. Hydrogen sulfide production test of StrainA_5



Figure 6. Starch hydrolysis test of StrainA_5



Figure 7. Carbohydrate Assimilation test of StrainA_5