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PRODUCTION AND EVALUATION 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. *In a* medium formulation study, A-5 *Actinomycete* mutant strains were evaluated for maximum antibiotic production using various carbon and nitrogen sources. Various fermentation conditions such as pH, temperature were also optimized for maximal production of antibiotic from A-5 strain. Also determined various physical properties of ethyl acetate extracted compound and MIC value against different strains of bacteria and fungi. Findings from this investigation reveal that strain A-5, in that order, exhibited superior antimicrobial activities to other soil isolates of *Actinomycetes*.

INTRODUCTION

Actinomycetes are the most widely distributed group of microorganisms in nature which primarily inhabit the soil[1]. They have provided many important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive compounds. *Actinomycetes* comprise an extensive and diverse group of Gram-positive, aerobic, mycelial bacteria that play an important ecological role in soil cycles. Many are well known for their economic importance as producers of biologically active substances, such as antibiotics, vitamins and enzymes[2,3,4,5]. In addition, they are one of the major communities of the microbial population present in soil, and their occurrence is greatly influenced by the environmental conditions of humidity, temperature, pH and vegetation. *Actinomycetes* have been well known for the production of secondary metabolite. Many of the presently used antibiotics such as streptomycin, gentamicin, rifamycin and erythromycin are the product of *Actinomycetes*. The *Actinomycetes* are important not just to the pharmaceutical industries but also the agriculture. Previous study showed that *Actinomycetes* isolated from Malaysia soil have the potential to inhibit the growth of several tested plant pathogens[6]. The ability of *Actinomycetes* isolated from Turkey's farming soil have the ability to inhibit *Erwinia amylovora* a bacteria that cause fireblight to apple and *Agrobacterium tumefaciens* a casual agent of Crown Gall disease[7]. Soil *Actinomycetes* for the most part show their optimum growth in neutral and slightly alkaline conditions, and isolation procedures have been traditionally based on this neutrophilic character. Previous works showed the existence of a large diversity of acidophilic *actinomycetes* that differed morphologically and physiologically from neutrophilic species[8,9]. Acidophilic isolates grow in the pH range 3·5–6·5, with optimum growth between pH 4·5 and 5·5; while neutrophilic strains grow in the pH range 5·0–9·0. Alkalophilic *Actinomycetes* are also known to occur in soils[10]. Many of these isolates, initially assigned to the genus *Streptomyces*, were later reclassified in the genus *Nocardiopsis* [11,13]. *Actinomycetes* can be isolated from soil and marine sediments. Although soils

have been screened by the pharmaceutical industry for about 50 years, only a small fraction of the surface of the globe has been sampled, and only a small fraction of *Actinomycetes* taxa has been discovered[14]. The present study was undertaken to isolate *Actinomycetes* from the soil samples of wasteland and garden of Coimbatore and to assess their anti-bacterial properties. The resistance problem demands that to discover new antibacterial agents effective against pathogenic bacteria resistant to current antibiotics. So we need to screen more and more *Actinomycetes* from different habitats for antimicrobial activity and fermentation in hope of getting some *Actinomycetes* strains that produce antibiotics that have not been discovered yet and active against drug resistant pathogens

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.

Optimization of fermentation parameters

Optimization of Carbon Source

From carbohydrate assimilation test, lactose was found to be a good carbon source for strain A_5. Different concentrations of lactose viz. 1%, 2%, 3%, 4%, 5% were used in the basal medium containing 0.3% yeast extract, 0.5% $CaCO_3$ and 0.25% NaCl separately in 250ml conical flasks at neutral pH and sterilized it. Then inoculated with the culture A_5 strain and kept on orbital rotary shaker (200rpm) at room temperature for 3 days. The quantity of the basal medium is 100ml. The

growth of micro organism which is determined by packed cell volume and quantity of antibiotic produced (antimicrobial activity) were measured after specific incubation period (72 hours). The concentration of lactose which gave maximum growth and yield of antibiotic was selected as carbon source for further studies.

Optimization of Nitrogen Source

Different sources of nitrogen such as peptone, yeast extract paste; ammonium chloride and ammonium sulphate were tested for maximum productivity of antibiotic and growth of organism. Initially 1% nitrogen sources were added to basal medium containing 4%lactose, 0.5% $CaCO_3$ and 0.25% NaCl at neutral pH in 250ml. conical flasks separately at neutral pH and sterilized it. Then inoculated with the culture A_5 strain and kept on orbital rotary shaker (200rpm) at room temperature for 3 days. The source of nitrogen which gave maximum growth and yield of antibiotic was selected as nitrogen source for further studies. The quantity of the basal medium is 100ml. The above process was repeated by adding the different concentrations (0.5%, 1%, 1.5%, 2%, 2.5%, and 3%) to basal medium containing 4%lactose, 0.5% $CaCO_3$ and 0.25% NaCl at neutral pH in 250ml conical flasks separately at neutral pH and sterilized it. Then inoculated with the culture A_5 strain and kept on orbital rotary shaker (200rpm) at room temperature for 3 days. The quantity of the basal medium is 100 ml. The concentration of peptone which gave maximum growth of micro organism and antibiotic production was selected as particular concentration of peptone.

Optimization of Temperature

Optimal temperature for production and growth of the strain A_5 was determined by keeping the inoculated fermentation media at different temperatures ranging from 25^0C, 27^0C, 29^0C, 31^0C, 33^0C, 35^0C up to 37^0C separately in 250ml conical flasks for 3 days in shaker incubator (200rpm). After incubation time, microbial growth and productivity of antibiotic were determined by measuring packed cell volume and antimicrobial activity. The optimum temperature giving

maximum productivity and growth was selected as optimal temperature. The quantity of the basal medium is 100 ml.

Optimization of pH

Optimum pH required for maximum productivity of antibiotic and growth of micro organism was determined by adjusting the pH of the fermentation medium at pH ranges between 5 to 8 separately in 250ml conical flasks and incubated at 27^{o}C for 3 days in shaker incubator (200rpm). pH is adjusted by 0.1N NaOH and 0.1N HCl. After, the incubation period, cultures were studied for its growth and productivity of antibiotic. The optimum pH which gave maximum packed cell volume and productivity of antibiotic was selected as optimal pH. The quantity of the basal medium is 100 ml.

Time course of fermentation process under optimum condition

The 250ml conical flask with 100ml basal medium containing 4% lactose, 1.5% peptone, 0.5% $CaCO_3$ and 0.25% NaCl at the pH 7 and temp. 27^{0}C inoculated with A_5 strain in shaker incubator (200rpm) for 6 days. After every 24 hour the culture broth was observed and monitored for the growth rate and productivity rate. At particular time period the growth rate and antibiotic production was maximum and Recorded this time period. A new batch was run in the 500ml basal medium by keeping all the optimized parameters at above set value. Basal medium divided into two equal parts and transferred into two 250ml conical flasks.

Downstream process for an antibiotic produced by A_5

The collected fermented culture was centrifuged at 10,000rpm for half an hour at 4°C and the antimicrobial activity was determined for the fermented broth (Supernatant) and the disrupted cell pellets. Since the cell pellet doesn't show any antimicrobial activity only the supernatant was subjected for further extraction and isolation of its active component by the following steps.

Extraction of antibiotic from culture supernatant by using different solvents

Divided the supernatant in 10ml four aliquots and four solvents were used and tested for the extraction of antibiotic from the culture supernatant. The solvents were used ethyl acetate, petroleum ether, methanol and chloroform.The solvents were added to the supernatant in 1:1 proportion. Solvent supernatant mixtures were shaken vigorously for 1hr. for complete extraction. The organic solvents layers from the above steps were subjected to the antibacterial study using respective solvents as control by agar well diffusion method. Organisms used were- *B. subtilis and E. coli.* Among four solvents layer, only ethyl acetate layer was found to show antibiotic property. Then the remaining fermented broths were also extracted with ethyl acetate. The ethyl acetate phase that contain antibiotic was separated from the aqueous phase by the separating funnel. It was evaporated to dryness in water bath at 80^0C -90^0C and residue obtained was weighed.

Determination of antimicrobial activity

Antimicrobial activity was determined by cup plate technique. The test bacteria were grown on nutrient agar while the test fungi were grown on Sabouraud dextrose agar. The extracts, i.e., supernatant liquid from fermentation media, were dissolved in 200 ml of ethyl acetate and 50 µL of the solution was placed in the agar cup. Growth inhibition was measured after incubation for 24 h at 37 ^{0}C for bacteria and 72 h at 27 ^{0}C for fungi. Antimicrobial activity was estimated by measuring the inhibition zone diameters [15].

RESULTS AND DISCUSSION

Optimization of fermentation parameters

Optimization of Carbon Source

From the carbohydrates assimilation test lactose was found to be the good carbon source for the growth of the strain A_5. To optimize the concentration of lactose for better growth and productivity different concentrations of lactose were described under Materials and Methods. By

determining its packed cell volume (PCV) and zone of inhibition of all the concentrations, Lactose of 4% was found to be the better source for maximum productivity of the strain A_5. The results are shown in Fig. 1.

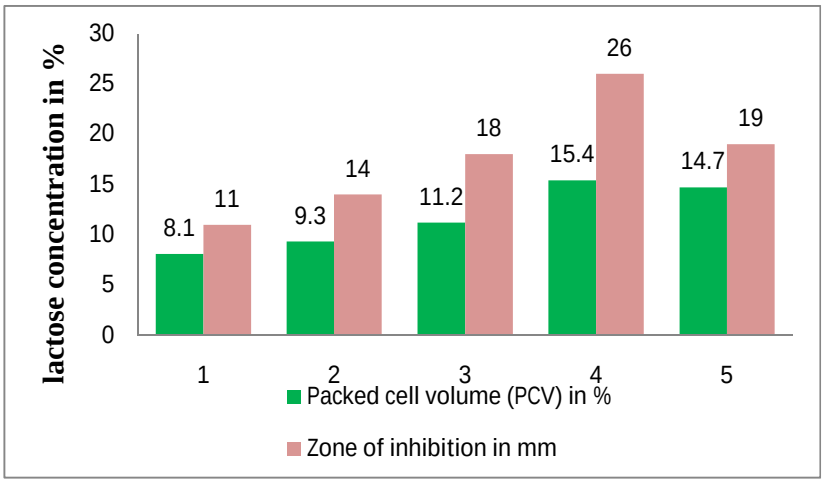

Fig. 1: Optimization of lactose as carbon source

Optimization of Various Nitrogen Sources

Among the four nitrogen sources only peptone found the best nitrogen source for the strain of *Actinomycetes* A_5 maximum growth and productivity. After then to optimize the concentration of peptone for better growth and productivity, different concentration were study and as described under material and method. From the results of determination of packed cell volume (PCV) and zone of inhibition, it was revealed that peptone 1.5% was found to be the good source of Nitrogen for the *Actinomycetes* A_5. The results are shown in Fig. 2 and 3.

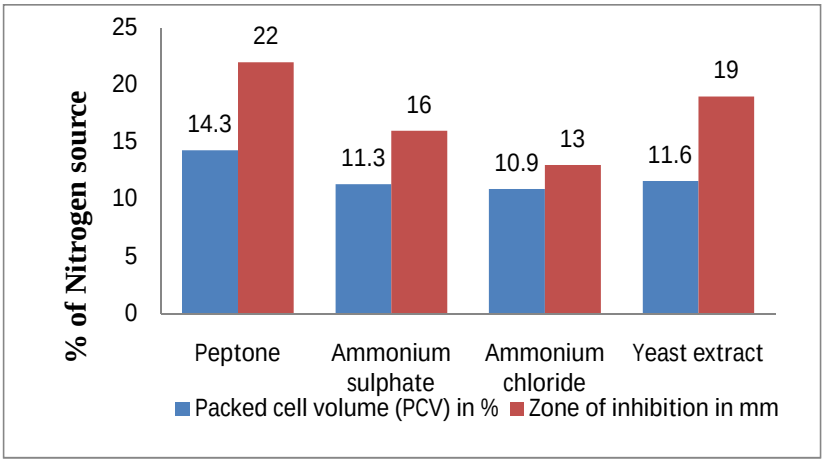

Fig. 2: Optimization of various nitrogen sources

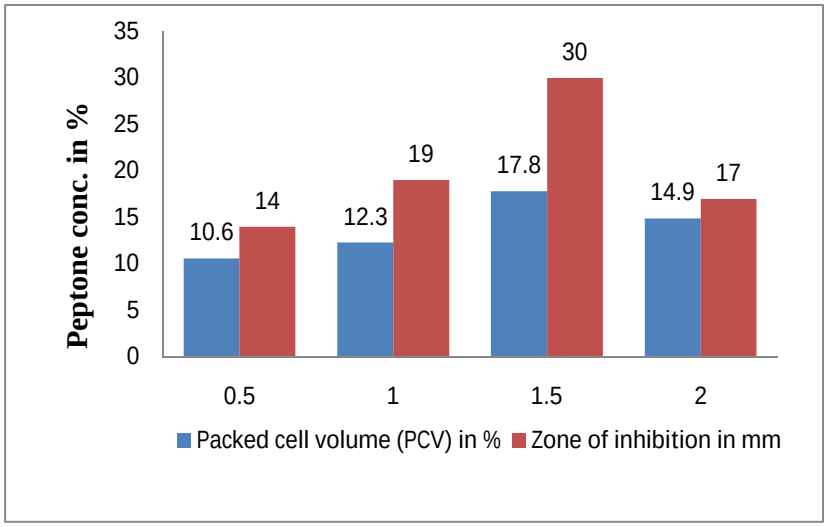

Fig. 3: Optimization of peptone as nitrogen source

Optimization of temperature and pH

Optimum temperature and pH for maximum growth and productivity of *Actinomycete* A_5 were determined by studying its packed cell volume (PCV) and zone of inhibition. From the results of determination of packed cell volume (PCV) and zone of inhibition, it were revealed that

temperature 27°C and pH 7 were found to be the suitable for the *Actinomycete* strain A_5. Results are shown in Table. 1 and 2.

Table 1: Optimization of Temperature

Temperature	Packed cell volume (PCV) in percentage	Zone of inhibition (Diameter in mm)
25°C	8.1	18
27°C	11.9	29
29°C	10.4	22
31°C	10.1	20
33°C	7.6	15
35°C	7.3	11
37°C	6.4	8

Table 2: Optimization of pH

pH	Packed Cell Volume (PCV) (%)	Zone of inhibition (mm)
5	7.7	19
5.5	8.2	21
6	8.7	24
6.5	9.1	26
7	12.7	30
7.5	10.2	27
8	8.8	21

Duration of Fermentation

Duration of fermentation for maximum growth and productivity of *Actinomycete* strain A_5 were determined by studying its packed cell volume (PCV) and zone of inhibition. By studying its packed cell volume (PCV) and zone of inhibition 96hr are suitable for maximum production of *Actinomycete* strain A_5. Results are shown in Table 3. Based upon the above-consolidated results, fermentation medium and fermentation parameters were optimized for the maximum production of antibiotic and they were given as follows. Composition of fermentation Medium was Lactose -4%, Peptone -1.5%, $CaCO_3$ -0.5%, $MgSO_4.7H_2O$- 0.025%, K_2HPO_4 -0.1 %, NaCl- 0.25% , $FeSO_4.4H_2O$ -0.001 % . Fermentation conditions- temperature-27°C, pH-7.0, duration of fermentation-4 days, inoculums concentration-100µl.

Table 3: Duration for Fermentation

Time in Hours	Packed Cell Volume (PCV) (%)	Zone of inhibition (mm)
0	-	-
24	2.2	19
48	9.7	23
72	14.9	32
96	19.1	34
120	18.8	31
144	17.1	30

Downstream process of antibiotic

The cells were separated from the fermentation broth by centrifugation. The supernatant was extracted with different solvents. Only Ethyl acetate layer was showing antimicrobial activity. Then the remaining fermented broth was also extract with ethyl acetate. The ethyl acetate phase that contain antibiotic was separated from the aqueous phase by the separating funnel. It was evaporated to dryness in water bath at 80^0C -90^0C. Results are shown in Fig. 4. Weight of ethyl acetate extracted antimicrobial compound was found to be 17.360g.

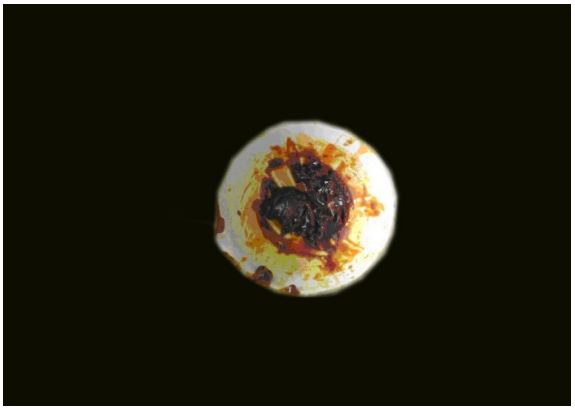

Fig. 4: Ethyl acetate extracted antimicrobial compound

Determination of antimicrobial activity

The antimicrobial activity in terms of minimum inhibitory concentration (MIC) of an isolated compound from A_5 fermented broth was studied. The MIC of antibiotic against gram positive and gram negative bacteria were found to be 2.5mg/ml. surprisingly the compound was found significant antifungal activity against two strains of fungi tested. The MIC was found to be 2.5 mg/ml for *Candida albicans* and 1.25mg/ml for *A. flavus*. Results are shown in Table 4.

Table 4: Determination of MIC for an isolated antimicrobial compound against different strains of Bacteria

Test organism of bacteria and fungi	**MIC value of A_5**
Bacillus subtilis	2.5 mg/ml
Escherichia coli	2.5 mg/ml
Candida albicans	2.5 mg/ml
A. flavus	1.25mg/ml

CONCLUSION

In the optimization of fermentation process, the optimization of different factors like carbon source, nitrogen source and different parameters like optimization of temperature, pH and duration of fermentation process were carried out. After running batch fermentation, the product obtained from it was taken for downstream processing. In extraction, different solvents were used and tested for the extraction of antibiotic from the culture supernatant. The solvents were used ethyl acetate, petroleum ether, methanol and chloroform. Among four solvents layer, only ethyl acetate layer was found to show antibiotic property. Then the remaining fermented broth was also extract with ethyl acetate. After downstream processing, determined the various physical properties of ethyl extracted antimicrobial compound. Colour of extracted antimicrobial compound is Dark orange and sticky semi solid at room temperature. It was soluble in water, n-butanol, methanol, DMSO, carbon tetra chloride. It was sparingly soluble in chloroform and insoluble in petroleum ether, ethyl acetate and benzene. The MIC values of antimicrobial ethyl extracted compound were found to be 2.5 mg/ml for both *B. subtilis and E. coli* respectively. For *C. albicans* it passed 2.5 mg/ml and for *A. flavus* passed 1.25mg/ml. The MIC is not a constant for a given agent, because it is affected by the nature of the test organism used, the inoculum's size, and the composition of culture media, the incubation time, and aeration. Optimization of other parameters such as effect of minerals, agitation speed, control of foam etc may help to increase the further productivity of the antibiotic in laboratory scale and pilot scale fermentation.

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