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CULTURAL, PHYSIOLOGICAL AND MOLECULAR CHARACTERIZATION OF ACTINOMYCETES FROM THE SOIL OF MUZAFFARPUR DISTRICT

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ABSTRACT
Actinomycetes constitute a formidable group of industrially important microorganisms that have been greatly explored for the production of secondary metabolites. The solid samples were collected from litchi garden, rhizoplane of herbaceous plants and water logged land. A total of 72 strains were isolated on seven culture media and cultural characteristics of these strains were observed. The viable count was found to be maximum (12.82×10^4) per gram dry weight of litchi garden soil on Czapek-Dox agar medium. Least population was recorded on water logged soil. Moderate to heavy sporulation was noticed in some of the strains. White and gray pigments remained dominant on all media used. The utilization of different carbon sources by these strains was reported. On the basis of pigment production, sporulation and luxuriant growth, eight strains out of 72 could be singled out, subcultured and purified in the laboratory conditions. Antagonistic effects especially against six antibiotics were noticed and some of the strains exhibited resistance to ampicillin, kanamycin and streptomycin. The evidence for the presence of plasmid was searched out on agarose gel electrophoresis and later electrophoretic separation of sharp bands on agarose gel was also observed.

INTRODUCTION

Actinomycetes are ubiquitous bacteria which are found abundantly in the soil. They resemble fungi to some extent but their cellular and genetic organizations are enough to be placed in bacteria. They are capable of forming branched hyphae with or without spores at certain stage of development. The actinomycetes are widely known for providing clinically important antibiotics (Agustine *et al.,* 2005) and industrially indispensable enzymes.

Actinomycetes form specific flora in the soil so they are abundantly found in garden soil rhizoplane of herbaceous plants and water-logged land (Miller *et al*., 1990). However, they fluctuate both qualitatively and quantitatively depending upon environmental conditions soil type and organic matter content (Abussaud and Saadoun, 1988). Viable counts per gram of dry soil can be obtained more from the garden soil. The variations in pH and temperature have definite role on the growth of actinomycetes. The number of colonies per plate for each medium varies because nutrient requirement for each species differs. Almost three-fourth of actinomycetes flora is dominated by *Streptomyces*.

Actinomycetes have also been extensively used in genetical studies because of several practical advantages such as their haploid nature, ease of handling and cultivation and a few of them with faster growth rate. Besides they play a vital role in the recycling of organic matters and nutrients in nature by decomposing tough plant materials like bark newspapers and woody stems. They find importance in microbial antagonism and in regulating the composition of soil community (Gesheva, 2001). The majority of actinomycetes are saprophytes, some species are pathogenic to animals and human beings while a few have symbiotic association with higher plants to fix atmospheric nitrogen.

The present study has therefore been undertaken to screen the actinomycete strains from the different sites of Muzaffarpur district and to characterize these strains on the basis of cultural and physiological parameters and sensitivity to different antibiotics. Further attempts were made to visualize the plasmids by these strains.

MATERIALS AND METHODS

Microorganisms

The soil is the most favourable place for the microbial population especially the actinomycetes. The soil samples were collected from litchi garden rhizoplane of herbaceous plants and water logged land of Muzaffarpur district by digging the soil upto 5-10 inches below the surface. These samples were taken in all seasons. The samples were stored into sterilized polythene bags and taken to laboratory for further processing (Goodfellow and O'Donnell, 1989; Hunter-Cevera and Eveleigh, 1990).

The strains were grown to different media and later exposed for their identification. The strains were later subcultured in both complete and minimal media. Thus isolated strains were purified on selective media for further investigation.

Composition of media

Several media were used for isolating the strains of actinomycetes from different sources. The pH of the medium was adjusted between 6.8 and 7.2 either by adding 1N NaOH or 1N HCl. Then the medium was subjected to autoclaving at 121ºC for 15 minutes. Seven different media were used for the isolation of actinomycetes:

1. Czapek-Dox Agar Medium consists of $NaNO_3$ – 2.0g, K_2HPO_4- 1.0g, $MgSO_4$-0.5g, KCl-0.5g, $FeSO_4 7H_2O$ -0.01g, Sucrose -30g, Agar-15g and Distilled water -1000 mL.
2. Actinomycetes Medium consists of $(NH_4)_2SO_4$ – 1.0g, K_2HPO_4 – 1.0g, NaCl -1.0g, $MgSO_4 7H_2O$- 1.0g, Starch - 1.0g and Tap water 1000 mL.
3. Soil Extract Agar Medium consists of K_2HPO_4-0.4g, $(NH_4)_2HPO_4$ – 0.5g, $MgSO_4 7H_2O$ – 0.05g, $MgCl_2$ – 0.01g, Peptone – 1.0g, Yeast extract – 1.0g, Agar - 15g and Tap water - 1000mL.
4. Glucose Asparagine Agar Medium consists of glucose – 1.0g, Asparagine – 0.5g K_2HPO_4 – 0.5g, Agar – 15g and Distilled water - 1000 mL.
5. Glycerol Asparagine Agar Medium consists of glycerol – 1.0g, Asparagine – 1.0g K_2HPO_4 – 1.0g, Agar - 15g and Distilled water - 1000 mL.
6. Starch Casein Agar Medium consists of soluble starch – 1.0g, Casein – 0.8g KNO_3 -2.0g, NaCl – 2.0g, K_2HPO_4 – 2.0g, $MgSO_4 7H_2O$ – 0.05g, $CaCO_3$ – 0.02 g $FeSO_4 7H_2O$ – 0.01g, Agar – 15g and Distilled water - 1000 mL.
7. Starch Agar Medium consists of soluble starch – 2.0g, Peptone – 5.0g, Beef extract – 3.0g, Agar - 15g and Distilled water - 1000 mL.

Normal saline

Normal saline was prepared by dissolving 8.7g of NaCl in one litre of distilled water. A drop of Tween- 80 was poured into it. It was then distributed in 15 mL vial containing 10 mL of normal saline and then subjected to autoclaving.

Serial dilution

A known amount (10 g) of soil is suspended or agitated in a known volume of sterile water blank (9 mL or so to make the total volume of 10 mL) to make a microbial suspension. Serial dilutions, 10^{-2}.....10^{-6} are made by pipetting measured volumes (usually 1mL) into additional dilution blanks having 9 mL sterile water. Finally 0.2 mL aliquot of various dilutions is added to sterile Petri plates containing 15 mL of sterile cooled and solid media. These plates were then subjected to incubation at desirable temperature. The number of colonies appearing on dilution plates are counted, averaged and multiplied by the dilution factor to find the number of cells/ spores per gram of the sample.

Cultural conditions

The isolate was characterized according to ISP procedure. The colours of mycelia and soluble pigment after growing on different media were observed by the naked eyes as well as the magnifying lens. To study the mycelia and its sporulation characteristics direct method and inclined cover slip methods were used.

Auxanography

Thick spore suspensions from the desired strains were mixed with cooled (45 – 50ºC) molten medium and then poured into sterilized Petri plates. The discs containing the different amounts of antibiotics such as ampicillin, gentamicin kanamycin, rifampicin, streptomycin and tetracycline were placed at specified marked position of the plates and then subjected to incubation for 48 h at 37 ± 2ºC. The zone of inhibition of these different antibiotics could thus be measured.

Isolation of plasmids

Plasmid of actinomycete strains was isolated by the methods of Birnboim and Doly (1979). Mycelia of actinomycete strains from a logarithmically (48 h) growing 40 mL cultures were harvested by centrifugation at 3000 x g for 10 min. The pellet was poured in 100 μL of solution I and vortexed gently. The sample was incubated at 37ºC for 30 min. Solution II was then added and immediately mixed by inverting the tube several times to obtain a clear viscous solution. The tubes were then chilled for 10 min on ice before adding 150µL of solution III. The tubes were further chilled for 1h at -20ºC to facilitate the precipitation of proteins RNA and DNA of the main genome. The mixture was centrifuged at 10000 x g for 20 min at 0ºC and then clear supernatant was collected.

Deproteinization was carried out twice by adding an equal volume of Sevag mixture. The tubes were then shaken for 10 min before centrifugation at 10000 x g for 20 min. The upper layer was carefully pipetted out and mixed with a double volume of chilled ethanol by inverting several times. The tubes were then kept overnight at -20ºC. The precipitated DNA in ethanol was centrifuged at 10000 x g for 20 min at 0ºC air dried and responded in 100 µL of solution IV. The amount of DNA was measured spectrophotometrically and electrophoresed.

Solution I: 2 mg mL^{-1} Lysozyme; 50 mM Sucrose; 10 mM EDTA (pH 8.0); 25 mM Tris-HCl (pH 8.0)

It was stored frozen and prepared weekly.

Solution II: 02N NaOH; 1% SDS;

It was freshly prepared

Solution III: 3 M Sodium acetate (pH 4.8)

Solution IV: 10 μM Tris-HCl (pH 8.0); 1 μM EDTA (pH 8.0)

Agarose gel electrophoresis

The method of Meyers *et al*. (1976) was followed. Electrophoresis was carried out in a horizontal gel tank (10 x7 cm) glass plate with about 3 mm thick agarose bed Agarose 1 % either in TBE or TAE buffer) was poured on glass plate. A comb with 8 teeth was placed over the agarose bed maintaining a distance of 0.05 mm between the glass plate and teeth of comb. The agarose was allowed to solidify for 60 min after which the comb was removed without disturbing the gel. The gel tank was filled with running buffer DNA samples (5 – 10 μL) were mixed with the tracking dye (2 – 4 μL) and loaded in the slots of the agarose bed with the help of a 20 μL micropipette. Initial 10 V of power was applied for 15 min which was raised to 60 V for 5-6 h or till the migration of the tracking dye to the far end. Electrophoresis was carried out at room temperature.

Running buffer

Tris - Acetate - EDTA buffer (TAE): 0.04 M Tris; 0.02 M Acetic acid; 0.02 M EDTA

Tris - Borate -EDTA buffer (TBE): 0.089 M Tris base; 0.02 M Acetic acid; 0.02 M EDTA

Tracking dye: 50% of glycerol of sucrose; 0.25% bromophenol blue; 0.1M EDTA

Staining of DNA of agarose gel

The method of Sharp *et al*. (1973) was followed. The agarose bed was immersed in a solution of ethidium bromide (0.5 μg mL^{-1}) and was kept for 45 min for staining it was then washed with running water. The DNA on gel was visualized by fluorescence on a Gel Documentation System (Genetix) at 254 nm. Photographs were taken through attached computer.

RESULTS AND DISCUSSION

The maximum number of actinomycete strains has been found in the soil of litchi garden due to the presence of great amount of organic matter and the minimum number has been noticed in the soil of water-logged land due to poor sporulation caused by high water activity (Ensign *et al*., 1986). The luxuriant growth of actinomycetes was observed in Czapek-Dox agar medium but other media were also found favourable for the isolation of strains (Table 1).

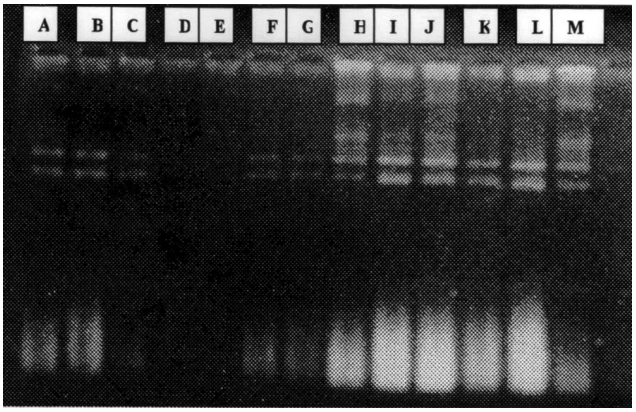

Figure 1: Electrophoretic Separation of Sharp bands on Agarose gel after isolation of plasmid from different strains of Actinomycete (Birnboim & Doly's Method)A & B = KS-10 (3 μg); C = KS-10 (1.5 μg); D & E = KS-60 (3μg)F & G = KS-22 (3 μg); H I & J = KS-30 (3 μg) K & L = KS-44 (3 μg)M = KS-65 (3 μg)

Table 1: Viable count of actinomycetes from different soil samples

Media used	Litchi garden	Rhizoplane of herbaceous plants	Water-logged land
Czapek-Dox Agar	12.82×10^4	10.72×10^4	8.48×10^4
Actinomycetes medium	10.60×10^4	8.32×10^4	5.82×10^4
Soil Extract Agar	6.68×10^4	4.92×10^4	2.32×10^4
Glucose-Asparagine Agar	$5.48x\ 10^4$	3.13×10^4	2.86×10^4
Glycerol-Asparagine Agar	3.42×10^4	2.32×10^4	2.26×10^4
Starch-Casein Agar	3.78×10^4	2.06×10^4	1.78×10^4
Strarch Agar	2.56×10^4	1.72×10^4	2.56×10^4

Table 2: Cultural characteristics of eight actinomycete strains on CDA medium

Strains	Colour of mycelia	Pigment	Sporulation
KS-10	Creamy	Absent	Heavy
KS-22	Bluish	Bluish	Heavy
KS-30	Brown	Grey	Heavy
KS-44	White	Absent	Heavy
KS-52	Grey	Brown	Moderate
KS-60	Creamy	Absent	Moderate
KS-65	Brown	Grey	Heavy
KS-72	Grey	Absent	Heavy

Table 3: Utilization of different carbon sources by actinomycete strains

	Growth of Strains from different sites		
Carbon sources	Litchi garden	Rhizoplane	Water-logged land
Glucose	+ +	+ +	+ + +
Galactose	+ + +	+	+ +
Fructose	+	+ +	+ +
Sucrose	+ +	+ +	+
Starch	+ +	+ +	+ +
Cellulose	+ + +	+ +	+ + +
Sorbitol	-	+	-
Glycerol	-	-	+
Sodium acetate	+ +	+ +	+
Sodium citrate	+ +	+ +	-

+ = Poor growth, + + = Moderate growth, + + + = Luxuriant growth, - = No growth

Out of 72 strains of actinomycete isolated on different media, 40 strains were found to have secreted the diffusible pigments of different colours ranging from creamy white to brownish gray (Ellaiah *et al*., 1996, 2002). The suitable temperature for the growth of most of the strains was found to be 37 ± 2ºC in case of mesophiles and 52 ± 2ºC for thermophiles (Barbban and Edwards, 1996: Edwards,1993) The optimum pH for growth was 6.8 but pH between 6.8 and 7.2 was also favourable (Peela *et al*., 2005; Sahin and Ugur, 2003)

Eight strains were purified by single colony method and were named as KS-10 KS-22, KS-30, KS-44, KS-52, KS-60, KS-65 and KS- 72.All were mesophiles except KS-52 and KS-60 which showed thermophilic response (Kokare *et al*., 2004a, 2004b; McCarthy, 1985). The cultural characteristics revealed that the strains KS-22, KS-30, KS-52 and KS-65 secreted bluish grey brown and grey diffusible pigments respectively with typical earthy odour on Czapek-Dox agar medium (Ishibashi, 1992) but the strains KS-10KS-44KS-60 and KS-72 did not secrete any pigment at all(Table 2).

The physiological characterization of these strains was based on the utilization of different carbon sources such as monosaccharides, oligosaccharides, polysaccharides and trihydric alocohol etc. The luxuriant growth of the strains of litchi garden soil was observed on the medium containing

Table 4: Zone of inhibition of actinomycete strains against different antibiotics

	Zone of inhibition (mm) after 48h					
Isolated strains	A	G	K	R	S	T
KS-10	26^s	20^s	11^r	26^s	14^r	32^s
KS-22	30^s	26^s	16^r	22^s	17^r	35^s
KS-30	24^s	16^r	22^s	23^s	26^s	32^s
KS-44	16^r	20^s	20^r	25^s	24^s	30^s
KS-52	18^r	24^s	22^s	24^s	26^s	30^s
KS-60	20^s	22^s	24^s	26^s	24^s	28^s
KS-65	22^s	20^s	22^s	24^s	22^s	26^s
KS-72	18^s	24^s	24^s	22^s	26^s	22^s

A = Ampicillin, G = Gentamicin, K = Kanamycin, R = Rifampicin, S = Streptomycin, T = Tetracycline, r = resistance, s = sensitive.

galactose and cellulose as carbon sources. The strains of rhizoplane showed heavy growth on the medium containing fructose. The luxuriant growth of the strains of water-logged soil was seen on the medium containing glucose and cellulose. Trihydric alcohols such as glycerol and sorbitol showed either poor or no growth of the strains (Table 3).

The antibiotic resistance profile of the eight isolated strains was studied against six antibiotics viz. ampicillin, gentamicin, kanamycin, rifampicin, streptomycin and tetracycline (Bonafede and Louis, 1997). The strains KS-10 and KS-22 were found to be resistant against kanamycin and sensitive against other antibiotics. KS-30 was found to be resistant against gentamicin and sensitive against others. The strains KS-44, KS-52 and KS-72 showed resistance against ampicillin and sensitive against others. The strains KS-60 and KS- 65 were found to be sensitive against all antibiotics tested (Haque *et al*., 1992; Table 4).

The antibiotic resistance was found to be different on solid medium than on liquid medium. It is because the rate is always slower on solid substrate than in liquid shake culture. Since the liquid shake cultures facilitate synchronous growth of mycelia the accurate and convenient way to monitor the growth profile against antibiotics continued to be through liquid culture (Siva Kumar *et al.*, 2005; Thomson *et al.,* 1982).

The evidence for the presence of plasmid was searched out on agarose gel electrophoresis and it was found that the extra chromosomal bands were clearly present on different strains once the technique for plasmid isolation was exposed (Sinha *et al.,* 1992). The gel electrophoresis demonstrated the presence of two distinct bands in the strains KS -10 and KS - 22 and three to four bands in strain KS-30 whereas there was no band in the strain KS-60. Examination of gels did not reveal any chromosomal contamination since chromosomal smear was not found in any of the slot in the vicinity of distinct bands on the application of plasmid preparation; these bands were naturally treated to be extrachromosomal in origin. The contamination of RNA and others could be observed at the end of each slot (Fig. 1).

On the basis of overall data obtained, following conclusion could be drawn. Seasonal variation did not have any significant influence on the growth of actinomycete strains. Post incubation refrigeration could be helpful to understand the influence of diffusible pigment under restricted growth conditions. The findings highlight the prevalence of strains in every type of soil even in water-logged soil. These isolates could be a choice to see the enzymatic activities especially cellulase from mesophilic strains.

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