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ISOLATION AND OPTIMIZATION OF CELLULASE PRODUCING ACTINOMYCETES FROM SOIL OF MEHSANA DISTRICT, GUJARAT

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ABSTRACT
Twenty eight actinomycetes strains were isolated from cotton plant rhizosphere soil of North Indian region of India. The isolates were identified as actinomycetes by morphological studies. Among them 11 strain are selected on basis of cellulolytic activities by primary screening. Cellulolytic activities were exhibited by strain A1, A2, A3, A8, A11, A13, A16, A18, Grey 72, AB1, AB4 and it was confirmed by formation of clear zone of hydrolysis around the colonies. Among the isolates maximum cellulolytic activity were exhibited by strains AB4 and it was determined by Enzyme assay.

KEYWORDS: *Actinomycetes*, Cellulolytic activities, Endocellulases, Exocellulases, *Carboxymethyl cellulose* (CMC).

INTRODUCTION

Actinomycetes are traditionally considered to be transitional forms between bacteria and fungi. Like fungi, they form a mycelial network of branching filaments, but like bacteria they are thin, possess cell walls containing muramic acid, have prokaryotic nuclei and are susceptible to antibacterial antibiotics[3,14]. They are therefore true bacteria, bearing a superficial resemblance to fungi. Actinomycetes are related to mycobacteria and corynebacteria. They are Gram-positive, nonmotile, nonsporing, noncapsulated filaments that break up into bacillary and coccoid elements [5,8,22]. Most are free living, particularly in the soil. They include the anaerobic Actinomyces, Arachnia, Bifidobacterium and Rothia species, and the aerobic Nacardia, Actinomadura, Dermatophilus and Streptomyces species[30]. The major pathogenic genus, Actinomyces is anaerobic or microaerophilic and non-acid fast, While the Nacardia species are aerobic and may be acid fast[8]. Species of Streptomyces may causes disease but their importance is as a source of antibiotics. Actinomyces bovis, probably the most common of the six species of Actinomyces, causes disease in cattle, while Actinomyces israelii and others cause disease in humans[22]. Generally, abscesses are produced in bone and soft tissue, with chronic draining sinuses to the exterior Sulphur granules are found in the exudate. The morphology in diseased tissue is of a long branching mycelium with attached 'Sulphur granules'. The Actinomyces species requires an anaerobic environment to be isolated from clinical specimens, but then can be maintained under microaerophilic conditions[1,7]. Treatment consists of surgical drainage or excision, together with large doses of penicillin for several weeks or month.

Degradation of cellulose by microorganism is a major component of the carbon and energy flux in soil. Among the microorganism soil actinomycetes have been a great source of new compound and enzymes[8,14,31]. Cellulose is a linear polysaccharides of glucose residues with β-1, 4-glycosidic linkages. *Actinomycetes* are one of the known cellulase producers [19, 20]. *Streptomyces drozdowiczii, S. lividans, S. longispororuber, S. rutgersensis, Streptomyces sp. B-PNG23* are better examples for production of cellulase and used in industries such as pulp and paper, textiles, bio refineries , animal feed stocks, wine and brewing, baking[21,22]. Alkaline or alkalitolerant and cellulase producers are mainly found in the genera *Streptomyces* and *Thermoactinomyces*[23]. Cellulolytic enzymes are employed in the color extractions of juices, in detergents causing color brightening and softening, in the biostoning of jeans, in the pretreatment of biomass that contains cellulose to improve nutritional quality of forage and in the pretreatment of industrial wastes[24,27]. Actinomycetes, a separate taxonomic group within domain bacteria, are members of the order Actinomycetes[29]. They are Gram positive bacteria, primarily aerobic and spore formers, with high G+C content[30]. As their name reflects (in Greek, "atkis" means ray and "mykes" means fungus), they share some morphological features with fungi[31]. They show filamentous growth, producing aerial or substrate mycelium. Actinomycetes are responsible for earthy smell of the soil[29]. *These* numbers are very large, and an inevitable nasuta that has been *found* in most *aquatic* and *terrestrial environments* worldwide [29,32] including mangroves and sea sediments [33]. They belong to both mesophilic and thermophilic groups[34], which broaden the range of habitats inhabited by them. Actinomycetes are known to produce an extensive range of bioactive compounds including various enzymes having multiple biotechnological applications. Five general types of cellulases based on the type of reaction catalyzed. Endocellulases (EC 3.2.1.4) randomly cleave internal bonds at amorphous sites that create new chain ends. Exocellulases or cellobiohydrolases (EC 3.2.1.91) cleave two to four units from the ends of the exposed chains produced by endocellulase, resulting in tetrasaccharides[6]

or disaccharides, such as cellobiose. Exocellulases are further classified into type I based on that work processively from the reducing end of the cellulose chain, and type II, that work processively from the nonreducing end. Cellobiases (EC3.2.1.21) or beta glucosidases hydrolyse the exocellulase product into individual monosaccharide. Oxidative cellulases depolymerize cellulose by radical reactions, as for instance cellobiose dehydrogenase (acceptor). Cellulose phosphorylases depolymerize cellulose using phosphates instead of water.

MATERIALS AND METHODS

Sample collection from rhizosphere area of soil in Mehsana District

The soil sample is collected from different sites in Kherva, Visnagar, Patan, and Mansa in Mehsana District in India. The samples were collected using sterile new zip lock bags using sterile spatula. The samples were transported to the laboratory for the isolation of actinomycetes.

Enrichment and Isolation of soil actinomycetes

One gram of sediment soil were transferred into 100ml of starch casein broth containing Calcium carbonate-0.02g, Ferrous sulphate -0.01g, Magnesium sulfate-0.05g, potassium nitrate-2g, potassium phosphate-2g, Sodium chloride- 2g, Soluble starch-10g, Casein-0.3g and incubate in rotary shaker 36˚C for 7days. Streaking on Starch casein agar further isolated colony were selected from SCA plate and subculture were maintained 4˚C until further use.

Screening of Cellulase producing actinomycetes

Screening of Cellulase producing actinomycetes by using Cellulose agar plate containing Meat extract-0.3g, Peptone-0.5g, Cellulose-1%, Agar-3%, PH-7 incubate plate 36 ˚C for 6days after incubation add Iodine solution in cellulose plate for observing zone of hydrolysis near colony surface of actinomycetes.

Morphological characteristic of selected actinomycetes colony

Actinomycetes isolates were inoculated on SCA media and incubated for 7 days at 36°C. The colonies were observed under a high-power magnifying lens and colony morphology was noted with respect to color, aerial and substrate mycelium and diffusible pigment of colony[26].

Biochemical characteristic of selected actinomycetes colony

After preliminary studies, the isolates which were found to be positive were selected for biochemical studies. Biochemical tests generally used are gelatin hydrolysis, starch hydrolysis, urea hydrolysis, acid production from different sugars, hydrogen sulfide production test, triple sugar iron (TSI) agar test, citrate utilization test, indole test, methyl red test, Voges-Proskauer test, and catalase test, oxidase test[25].

Effect of temperature on enzyme activity

Selected isolates were streak on SCA and incubate at different temperature like 25°C, 30°C, 32°C, 34°C, 36°C, 38°C and 40°C for 72hrs after incubation diameter of zone of hydrolysis was measured [35].

Effect of pH on enzyme activity

Selected isolates were streak on SCA and incubate at different pH like 6, 6.5, 7.0, 7.5, 8.0, 8.5, 9.5 and 10 using 1N HCl and NaOH were adjusted. After 72hrs after incubation diameter of zone of hydrolysis was measured[35].

Effect of *Nacl* on enzyme activity

Selected isolates were streak on SCA and incubate at different *Nacl* concentration 0%, 1%, 1.5%, 2%, 2.5%, 3% and 3.5% using Nacl. After 72hrs of incubation diameter of zone of hydrolysis was measured [35].

Enzyme assay

Cellulase activity was measured by using 1% Cellulose as enzyme substrate. The reaction mixture containing 1ml of substrate solution of 0.25g of Cellulose make final volume 24ml of Distilled water in beaker and covered with aluminum foil and boiled to dissolve put in Water bath incubator shaker at 70°C for 20min. after 10min cooled down at room temperature. Addition of 0.5ml of culture supernant crude enzyme and 1ml of Sodium acetate buffer. The mixture was incubated at 37°C in Water bath incubator shaker at 120rpm for 30min. Released sugar was measured using 3, 5-Di nitro salicylic acid (DNSA reagent) and glucose as standard. The color was developed by boiling for 5min. take OD at 540nm [18].

RESULTSIsolation of soil actinomycetes

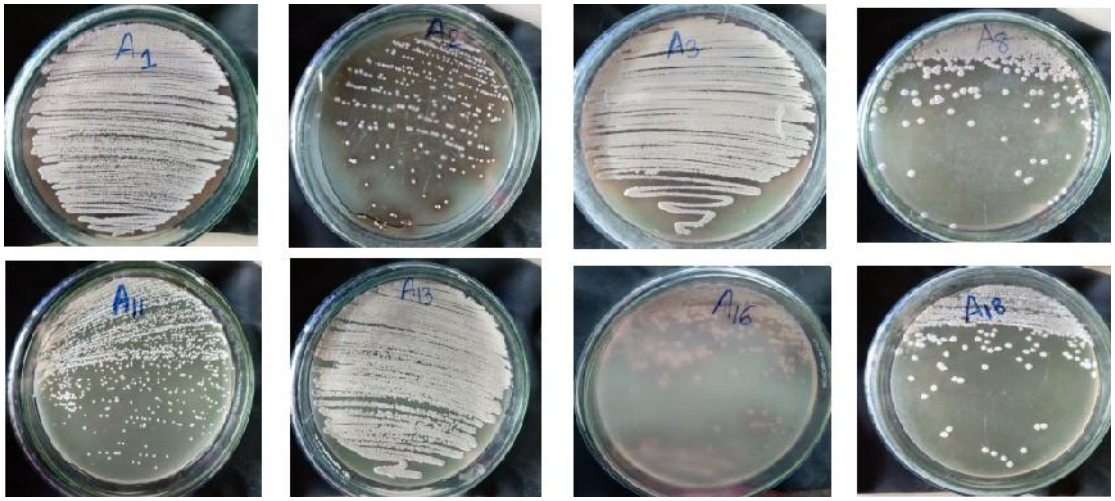


FIGURE 1 Isolation of soil actinomycetes

Screening of Cellulase producing actinomycetes

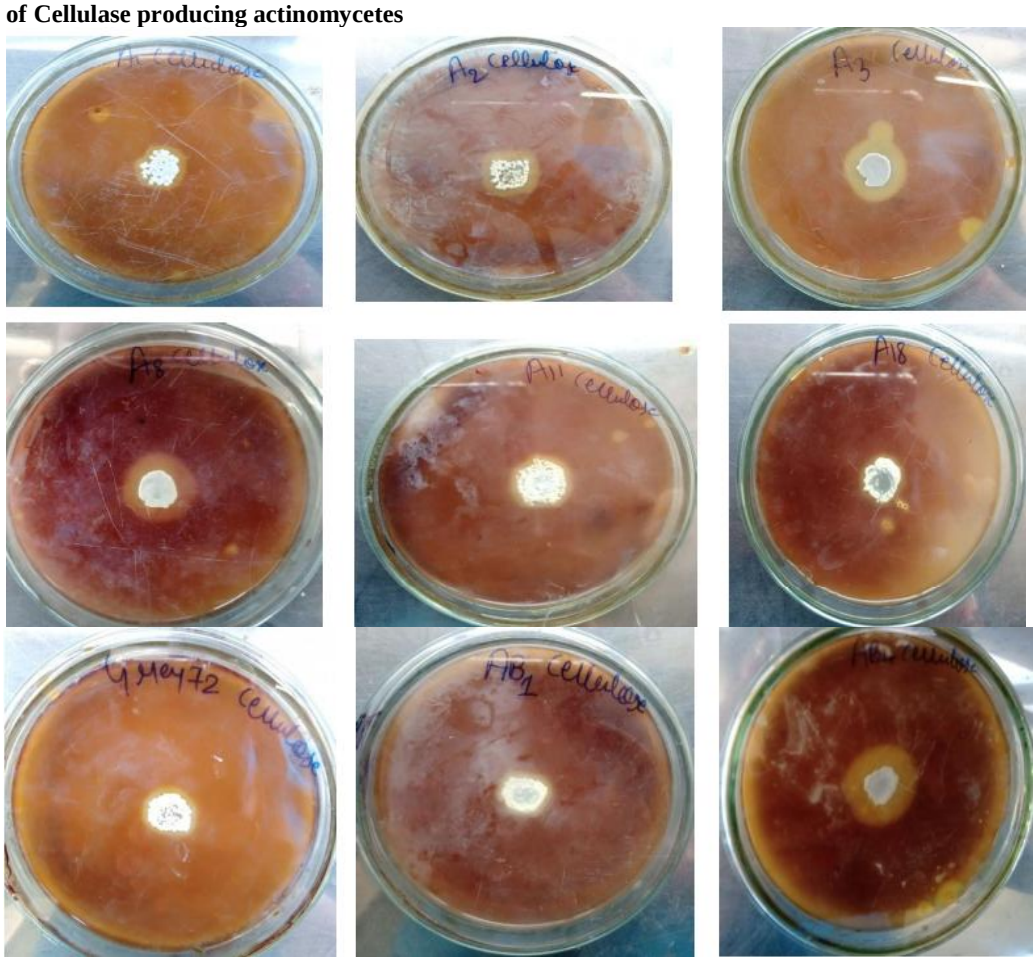
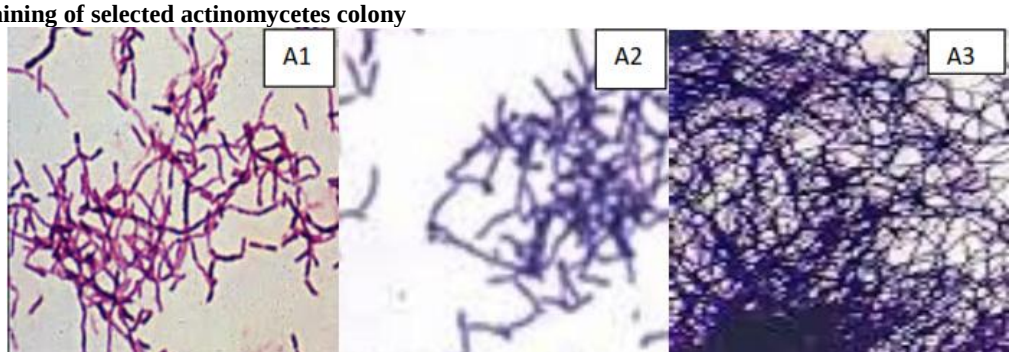


FIGURE 2 Screening of Cellulase producing actinomycetes

Gram's staining of selected actinomycetes colony



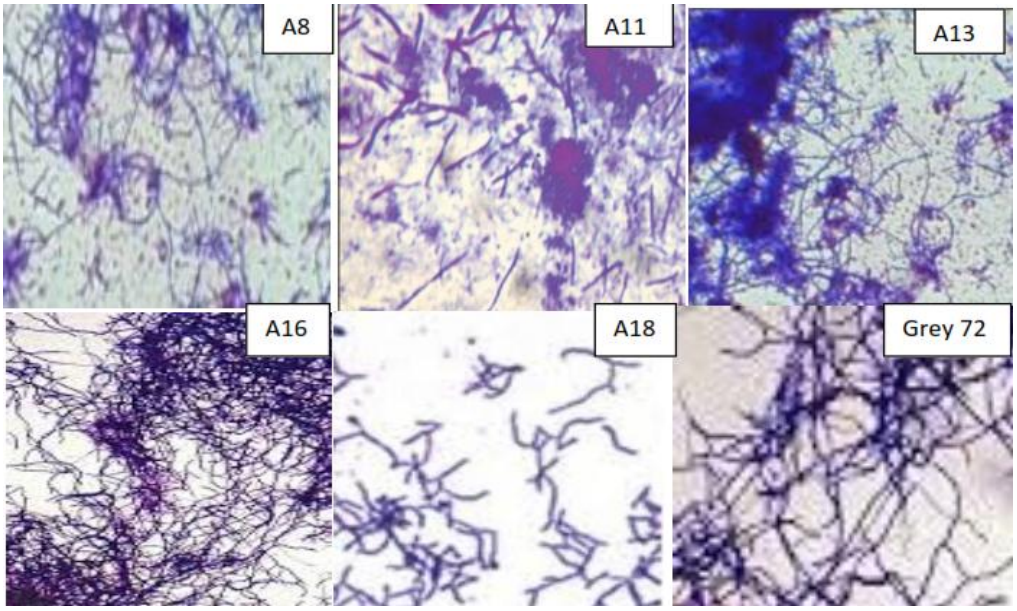


FIGURE 3: Gram's staining of selected actinomycetes colony

TABLE 1 Morphological characteristics of selected actinomycetes colony

Morphological characteristic of selected actinomycetes colony					
Isolates	Size	Aerial mycelium	Substrate mycelium	Diffusible pigment	Gram-staining
AB4	medium	white	Light-yellow	yellow	Gram negative
AB1	medium	white	brown	brown	Gram negative
Grey-72	Small	grey	white	non	Gram negative
A1	Small	white	Dark-pink	Ruby-red(red+pink)	Gram negative
A2	Small	white	Light-brown	Light-brown	Gram positive
A3	Small	Light-grey	brown	Brown	Gram positive
A8	medium	white	yellow	Yellow	Gram negative
A11	Small	white	Yellow	Non	Gram positive
A13	Small	Off-white	yellow	Yellow	Gram negative
A16	medium	brown	brown	Light-brown	Gram negative
A18	medium	white	white	Non	Gram negative

Biochemical characteristic of selected actinomycetes colony

TABLE 2 Biochemical characteristic of selected actinomycetes colony

Sr.no	Name of Test	AB4	AB1	Grey72	A1	A2	A 3	A8	A11	A13	A16	A18
1	Methyl red test	+	-	+	+	-	+	+	-	+	+	+
2	Voges–Prauskar test	+	+	+	-	+	+	-	+	+	+	+
3	Indole Production test	+	-	+	+	+	-	+	+	-	-	+
4	H2S production test	+	+	+	+	-	-	-	+	-	+	+
5	Nitrate Reduction test	+	+	+	-	+	+	-	-	+	-	-
6	Urea hydrolysis test	+	-	-	-	-	-	+	-	-	-	-
7	Citrate utilization test	-	+	-	-	-	-	-	+	-	-	-
8	Phenylalanine deaminase	-	-	+	+	-	+	+	-	-	+	-
9	Fermentation test											
	Glucose	+	+	+	+	+	+	+	+	+	+	+
	Dextrose	+	+	+	+	+	-	-	-	-	-	-
	Mannitol	+	+	-	+	+	-	+	+	+	+	+
	Fructose	+	-	-	-	-	+	-	-	-	-	-
	Maltose	-	+	+	+	+	+	+	-	+	+	-
	Sucrose	-	-	-	-	-	-	-	+	-	-	-
10	Amylase	+	-	+	+	+	+	+	+	-	+	-
11	Protease	+	+	+	+	+	+	+	+	+	+	+
12	Cellulase	+	+	+	+	+	+	+	+	+	+	+
13	Pectinase	+	+	+	+	+	+	+	-	-	-	+
14	Lipase	+	+	+	+	+	+	+	+	+	+	+

(+) indicates positive results (–) indicates negative results

Optimization of Enzyme activity

Effect of temperature on enzyme activity

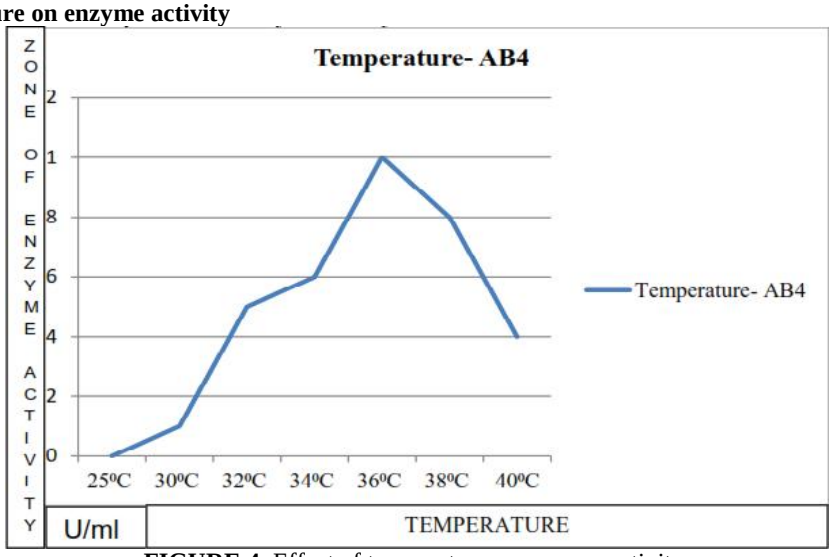


FIGURE 4: Effect of temperature on enzyme activity

Effect of temperature on enzyme activity: Among the different incubation temperature 35 C was found to be optimum to isolates species for the production and activity of the enzyme. The maximum production and activity of the enzyme was 21mm diameter of zone

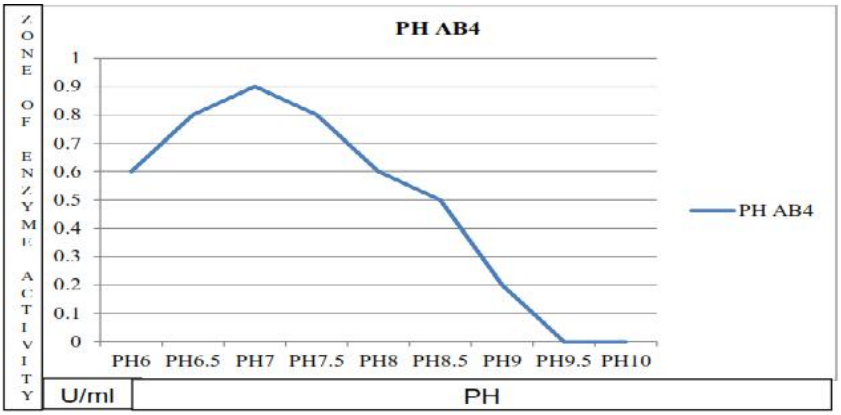


FIGURE 5 Effect of PH on enzyme activity

Effect of PH on enzyme activity: Among the different incubation PH 7 was found to be optimum to isolates species for the production and activity of the enzyme. The maximum production and activity of the enzyme was 20 mm diameter of zone.

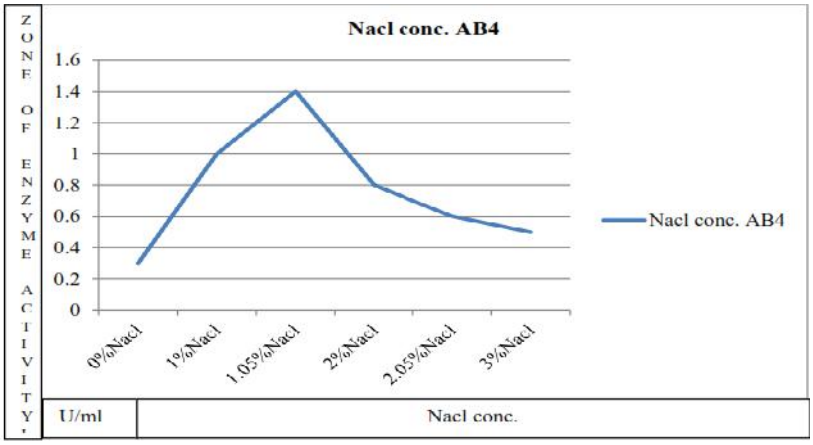


FIGURE 6: Effect of Nacl conc. on enzyme activity

Effect of Nacl on enzyme activity: Among the different incubation 1.5% was found to be optimum to isolates species for the production and activity of the enzyme. The maximum production and activity of the enzyme was 23mm diameter of zone.

Enzyme assay of isolated actinomycetes AB4:

TABLE 3 Enzyme assay of isolated actinomycetes AB4

Isolates	Substrate	Activity (U/ml)	Specific activity (U/ml)
AB4	Cellulose	30.12	37.65
	Carboxymethyl cellulose (CMC)	65.56	81.95

DISCUSSION

In the present study eleven actinomycetes were isolated from the rhizosphere by use of Enriched medium Starch casein broth for primary screening of actinomycetes. Selected actinomycetes from broth further streaking on Starch casein agar and incubated plates. Enrichment and Isolation of soil actinomycetes. one gram of sediment soil were transferred into 100ml of starch casein broth containing Calcium carbonate-0.02g, Ferrous sulphate - 0.01 g, Magnesium sulfate-0.05 g, potassium nitrate-2 g, potassium phosphate-2 g, Sodium chloride- 2g, Soluble starch-10g, Casein-0.3g and incubate in rotary shaker 36 ˚C for 7days. Streaking on Starch casein agar further isolated colony were selected from SCA plate and subculture were maintained 4 ˚C until further use. Morphological characteristic of selected isolates are observed based on size, aerial mycelium, substrate mycelium, diffusible pigment and Gram's staining. Most of the isolated actinomycetes gave white and grey aerial mycelium, yellow color gave substrate mycelium. Isolates AB1, AB4, A1, A2, A3, A8, A11, A13, A16 and A18 gave diffusible pigment while Grey-72 not gave diffusible pigment. After 7days incubation performs Gram's staining of selected actinomycetes colony was performed by use of Crystal violet, Gram's iodine, alcohol and safranin. Observed slide under 100X oil immersion lens. Isolated actinomycetes cell wall are stain with purple color due to crystal violet so isolated actinomycetes are Gram's positive actinomycetes. By performing biochemical test result are shown in table-2. Based on primary screening on cellulose agar plate select AB-4 which gave high cellulase activity by observing zone of hydrolysis on cellulose agar plate. Further study on AB4 optimization of enzyme activity by use of different parameters like temperature, PH and Nacl. Effect of temperature on enzyme activity Selected isolates were streak on SCA and incubate at different temperature like 25°C, 30°C, 32°C, 34°C, 36°C, 38°C and 40°C for 72hrs after incubation diameter of zone of hydrolysis was measured. Among the different incubation temperature 35 C was found to be optimum to isolates species for the production and activity of the enzyme. The maximum production and activity of the enzyme was 21mm diameter of zone for cellulase activity. Effect of pH on enzyme activity. Selected isolates were streak on SCA and incubate at different pH like 6, 6.5, 7.0, 7.5, 8.0, 8.5, 9.5 and 10 using 1N HCl and NaOH were adjusted. After 72hrs after incubation diameter of zone of hydrolysis was measured. Among the different incubation PH-7 was found to be optimum for cellulase activity of enzyme. The maximum production and activity of the enzyme was 20mm diameter of zone for cellulase activity. Effect of *Nacl* on enzyme activity. Selected isolates were streak on SCA and incubate at different Nacl concentration 0%, 1%, 1.5%, 2%, 2.5%, 3% and 3.5% using Nacl. After 72hrs of incubation diameter of zone of hydrolysis was measured. Among the different incubation 1.5%Nacl was found to be optimum for Cellulase activity of the enzyme. The maximum production and activity of the enzyme was 23mm diameter of zone for cellulase activity.

Authors Contribution statement

Mrs. Bhoomi Patel, Dr. Priti Patel and Mrs. Vaidehi Patel conceptualize and gathered the data with practical work experience done in Mehsana Urban institute of science laboratory, Kherva Mehsana Gujarat. With regard to this **"Isolation and optimization of cellulase producing actinomycetes from soil of Mehsana District, Gujarat".**

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