OPTIMIZATION OF CULTURE MEDIA FOR CELLULASE PRODUCTION FROM STREPTOMYCES RE11 ISOLATED FROM KALPAKKAM MANGROVE HABITAT

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ABSTRACT

Introduction:

Cellulase have diversified applications in the various industry. The natural and agricultural waste contains insoluble cellulose may be degraded through microbial degradation. We aim to isolate the maximum yield of enzyme from microbial source.

Materials and method:

The sediment water samples from Kalpakkam mangrove Habitat were processed for Actinomycetes strains isolation. The strains were evaluated for the preliminary screening for the production of cellulase enzyme. The effective strain was processed for the quantitative analysis of the cellulase production and characterized.

Results:

Totally 24 actinomycetes strains were cultured with different morphological characteristics and named RE1 to RE24. Only 5 strains were reported positive for cellulase production process. The maximum enzyme producer is RE11 (60.2 U/ml) compared with other 4 strain (RE22 (38 U/ ml) < RE05 (47.4 U/ml) < RE20 (49.4 U/ ml) < RE12 (58.2 U/ml)) and the strain was characterized as *Streptomyces pactum* according to the evolutionary relationship of 16SrRNA sequencing. With the optimized culture media the strain *Streptomyces pactum* was inoculated and the enzyme production was 73.6 U/ ml. Further the enzyme was partially purified using the optimized media.

Conclusion:

Hence it is concluded that the strain *Streptomyces pactum* was effective in the production of cellulase enzyme.

Keywords: Actinomycetes, *Streptomyces pactum*, Cellulase.

INTRODUCTION

All of the agricultural residues are collectively referred to as agricultural waste. It contains organic residues from a variety of agricultural activities, such as leftovers from industrial or agricultural processing, kitchen wastes, insects, straws, and animal and human excrement. Compost, fuel, and feed preparation were the main uses for agricultural waste, which contains inedible plant parts. These days, agricultural wastes are abandoned due to their low nutritional value, low calorific value, and refractory residues. The primary constituent of plant biomass and the most prevalent organic compound in the biosphere is cellulose, an insoluble polymer made up of lengthy chains of β-1,4-linked glucose residues arranged in microfibrils. Combinations of complementary microbial cellulases and other enzymes can break down plant cellulose to produce soluble sugars.

In the world's tropical and subtropical regions, mangroves are special types of intertidal ecosystems that sustain diverse groups of aquatic and terrestrial organisms with different genetic makeup. Mangroves are found along about 60–70% of the world's tropical and subtropical coastlines. These ecosystems are highly productive and have significant ecological value. These ecosystems are extremely productive everywhere in the world, despite their fragility and sparse distribution [Thatoi et al., 2008].

Mangrove ecosystems' microbial diversity can also reveal details about their ecological function and special biotechnological potential in the fields of industry, agriculture, pharmaceuticals, and medicine [Lageiro et al., 2007].

Because of their great production capacity, low cost, and genetic manipulation susceptibility, microorganisms are essential to enzymatic production processes. Microbial enzymes are of great biotechnological interest in a variety of fields, such as molecular biology, food processing, detergent and textile production, pharmaceutical and agricultural research, and medical therapy [Stamford et al., 1998]. [Carrim and others, 2006] (2011) Quecine et al. [Et al., Ferreira (2012)].

One of the most significant product categories is enzymes, which are made in factories using bacteria. Because bacteria are easily cultivated on the least expensive nutritional media and can be processed downstream with ease. The industrial production of enzymes like protease, cellulase, amylase, xylanase, lipase, pectinase, and peroxidase frequently uses actinomycetes. In order to ensure improved performance of the enzyme, such as high productivity, specificity, and stability at extreme temperature, pH, and inhibitor tolerance, existing enzymes are therefore engineered for new physical and physiological characteristics [Prakash et al., 2013]. The enzyme's capacity to scarify starch is also most widely used in the aforementioned industries. Using Actinomycetes as their extracellular enzyme, amylase, for instance, is the most useful enzyme in the food, pharmaceutical, textile, and brewing industries. It is produced industrially. Streptomyces erumpens's thermophilic and acidophilic amylases are particularly useful in the beer, bakery, and alcohol industries [Selvam et al., 2011].

The ability of cellulases to biodegrade cellulose into soluble sugars is crucial for the carbon cycle. These days, cellulases are also employed in the production of industrial ethanol and biofuel. Other than being used in the industrial production of cellulase, actinobacteria such as Streptomyces sp., Thermobifida halotolerans, Thermomonospora sp., and Streptomyces rubber are employed as detergents in the textile industry to remove stains, finish, and soften cotton, and in the paper and pulp production process to deink and modify fibers [Prakash et al., 2013].

The majority of research has concentrated on the cellulase enzyme because, according to Angelo (2004), it is the second most widely used class of carbohydrates because of its high degradation efficiency and specificity. Bioenergy production may benefit from the conversion of cellulose into simpler carbon forms [Bisaria VS & Ghose 1981]. Three different types of enzymes are generally induced to break down cellulose: endo-1,4-β-D-glucanase (also known as endocellulase), exo-1,4-β-D-glucanase (also known as exocellulase), and β-glucosidase (also known as cellubiose hydrolase). Endoglucanases, also known as endocellulases, are a class of cellulases that cleave random sections of cellulose to produce oligosaccharides of varying sizes. Exocellulases, also referred to as exoglucanases or cellodextrinases, function by releasing glucose or cellobiose at the ends of oligosaccharide chains that endocellulases have produced. Therefore, oligosaccharides are released from cellulose microfibrils when the glycosidic bonds in the microfibrils are broken by cellulases, increasing the digestibility of cellulose [Dillon A 2004]. Our goal is to separate the new strain from the mangrove ecosystem so that the cellulose enzyme can be assessed and purified.

MATERIALS AND METHODS:

Sample collection and pre treatment

Sediment and water samples were collected from Kalpakkam mangrove Habitat of Latitude: 12.511962°S'' Longitude: 80.160579°E'' with aseptic manner. The collected samples were transferred to the lab for the isolation and screening of cellulose producing organism. The physical parameters were evaluated for the samples like pH, Colour, odour and temp. The samples were pre-treated for further isolation process.

Isolation of Marine Actinomycetes:

One gram of sediment samples was suspended in 10 ml of sterile water and these suspensions were serially diluted. The dilutions were inoculated on SCA, ISP-5, MNA media added with Nystatin to minimize fungal contamination and Nalidixic acid to prevent Gram negative bacteria. All the plates were incubated at room temperature (28 ± 2°C) for three weeks.

The appearance and growth of colonies were observed every day and the colonies were recognized by their characteristic appearance. Totally twenty-four organisms were isolated and named RE 1, RE 2 and RE 3 to RE 24 respectively [Thenmozhi M & Kannabiran 2010[b]].

Qualitative assay for cellulose enzyme activity:

The isolated strain was grown on Carboxy methyl cellulose (CMC) agar plate and incubated. The presence of cellulase enzyme producing organism was confirmed by flooding the plate with 0.1% Congo red and washed with 1M NaCl. The presence of the clear zone around the colonies represents the enzyme producing organism [SangJoon et al., 2009].

Quantitative assay for cellulose enzyme activity:

About 200µl of culture was taken, to that 1800µl of 0.5% CMC prepared in 50mM sodium phosphate buffer (pH-7) was added. Incubate the reaction mixture in room temperature for 30minutes, then add 500µl of Dinitro salicylic acid (DNSA) reagent. The solutions were kept in boiling water bath for 5 minutes. Cooled down the reaction solution on completely, and the volume was made up to 5 ml using deionized water. Absorbance was measured at 575nm and the readings were observed. The same mixture except carboxy cellulose methyl was used as blank. One unit of cellulase activity is defined as the amount of enzyme that releases 1 µg amino acid equivalent to glucose per minute under the standard assay conditions [Wang et al., 2008].

$$\text{Units/ml} = \frac{\mu \text{ mole of glucose} \times \text{reaction volume}}{\text{Sample Vol} \times \text{reaction time} \times \text{Vol assay}}$$

Characterization of effective strain:

The effective strain were characterized for morphological, physiological (Carbon sources, Nitrogen Sources, pH, Temperature) biochemical and molecular characterization [Srinivasan et al., 1991]. The Biochemical characterization of the isolates include by evaluating the indole production, Methyl red production, Voges proskaur, Citrate utilization oxidase, triple sugar iron, TSI, Urease, Strach hydrolysis, catalase, Nitrate, Ammonium sulfate precipitation. The optimized condition was defined. The molecular characterization of effective strain done by extracting DNA and followed by PCR for characterizing 16S rRNA sequencing [Thenmozhi M and K. Kannabiran 2010[a]]. Phylogenetic trees was constructed using the neighbour joining method. Bootstrap analysis was done based on 1000 replications. The MEGA4 package was used for analysis.

Purification of cellulase Enzyme:

The isolate RE11 was cultivated in optimized production medium and kept for incubation. After complete growth it was centrifuged at 5000rpm for 10 minutes and the supernatant was collected. The total protein was precipitated at 85% saturation. The precipitate formed was collected by centrifugation at 10000rpm for 10 minutes. The pellet was dissolved in phosphate buffer at pH 7. The collected protein was dialysed overnight with 0.1m phosphate buffer and stored at 4°C. The cellulase enzyme production was checked by standard cellulase assays. The produced cellulase was partially purified.

SDS-PAGE analysis of the culture filtrate and ammonium sulphate precipitated crude protein was analysed [Senthil Kumar & Selvam 2011]. The determination of enzyme activity, the gel analyzed for zymogram.

RESULTS AND DISCUSSION

Sample Characterization:

The collected sample from Kalpakkam mangrove Habitat's physical characterization was observed and recorded. The colour of the sample was dark, the consistency was similar to clay, pH was identified as 5.5 (Fig. 1).

Fig 1. Sample collected from Kalpakkam mangrove Habitat

Marine Actinomycetes Isolation:

The serial dilution of the sample and plated for isolation of Actinomycetes. The morphologically different Actinomycetes species were identified and sub-cultured (Fig 2.). There was 24 isolates with different morphological features it was named as RE 1, RE 2, RE 3 … to RE 24 respectively for further identity. All these 24 isolates were used for primary screening process for enzymatic assay.

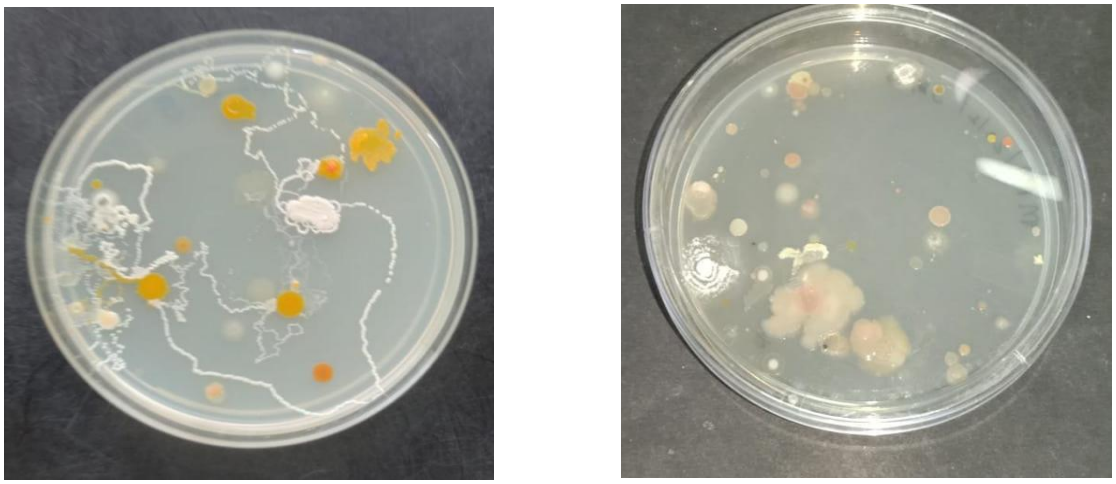

Fig 2. Serial dilution plates of isolates.

Preliminary screening for cellulase assay of isolated Actinomycetes:

The qualitative assay for the cellulase production using all the 24 isolates were evaluated (Table 1). Out of these isolates 5 strains were received positive for the cellulase production. The remaining strains were failed to produce the cellulase enzyme (Fig. 3). The active strains are RE5, RE11, RE12, RE20 and RE22.

Table1. The Qualitative assay of cellulase production for isolated strains.

Isolates	Cellulase
RE1	-
RE2	-
RE3	-
RE4	-
RE5	+ve
RE6	-
RE7	-
RE8	-
RE9	-
RE10	-
RE11	+ve
RE12	+ve
RE13	-
RE14	-
RE15	-
RE16	-
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RE18	-
RE19	-
RE20	+ve
RE21	-
RE22	+ve
RE23	-
RE24	-

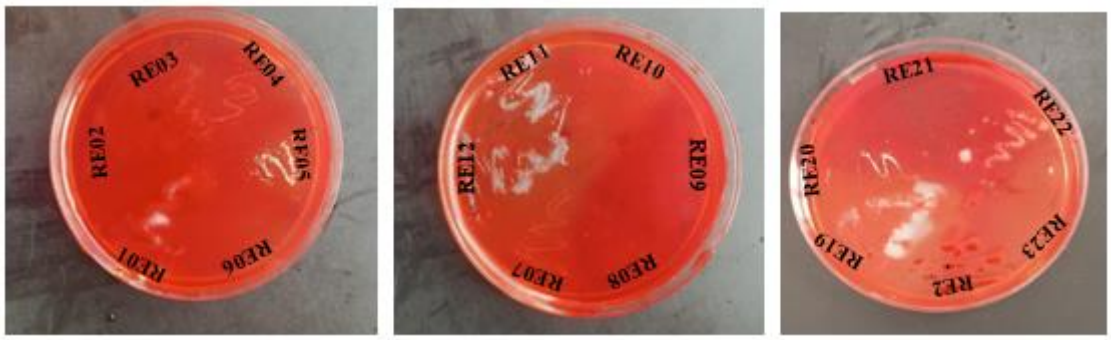

Fig. 3. Qualitative assay for cellulase production

The effective five isolates were measured spectrophotometrically at 550nm, where cellulose acts as a substrate. In this assay one unit of enzyme activity is defined as the amount of enzyme that liberated one µmol of glucose per minute under the specified conditions from the appropriate substrate.

The cellulase production of the selected five isolates in the following order RE22 (38 U/ ml) < RE05 (47.4 U/ml) < RE20 (49.4 U/ ml) < RE12 (58.2 U/ml) < RE11 (60.2 U/ml) (Fig. 4). The best enzyme producer were found to be RE 11 which produces 60.2 U/ml.

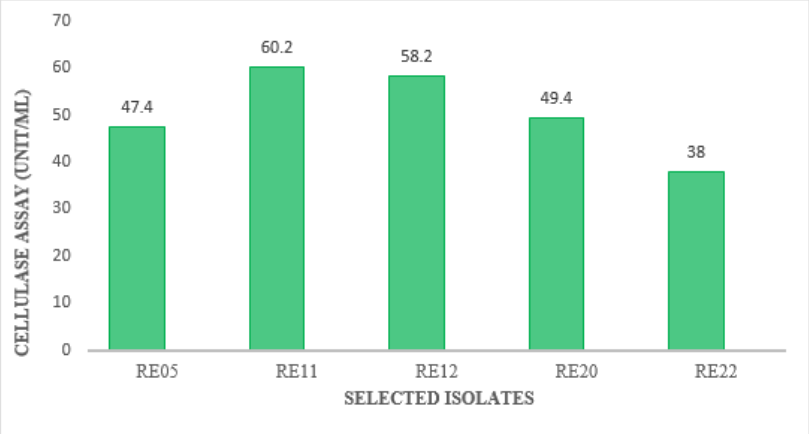

Fig 4. Quantitative assay for the cellulase production

Characterization of isolate RE11:

Among the five active Actinomycetes isolates RE11 was taken for further characterization (Fig.5). The morphological characterization represents white powdered colonies with white aerial mycelium and orangish red substrate mycelium. The microscopic staining results that gram positive rod shape. The optimized media for the enzyme production and suitable conditions were predicted.

Fig 5: The Actinomycetes isolate RE11.

The physiological characterization with the parameters such as temperature, pH, different carbon sources, nitrogen sources and which influence the secretion of cellulase enzyme by marine bacteria were optimized for maximum production. Among the different carbon sources tested (glucose, sucrose, CMC, starch and glycerol) glucose showed better activity of 30.6 U/ml (Fig 6). Among the different nitrogen tested (urea, ammonium sulphate, peptone, beef extract and yeast extract) yeast extract showed better activity of 78.6 U/ml (Fig 7). Among the various pH (5, 6, 7, 8 and 9), pH 8 showed better activity of 70.4 U/ml (Fig 8). The optimized culture media contained selected isolates was tested for cellulase activity showed good activity at 35°C with 73.4 U/ml (Fig 9). Both the optimized medium and control was used to check the cellulase enzyme production. Almost two times production was achieved after the optimization process which is 73.6 U/ mL (Fig. 10).

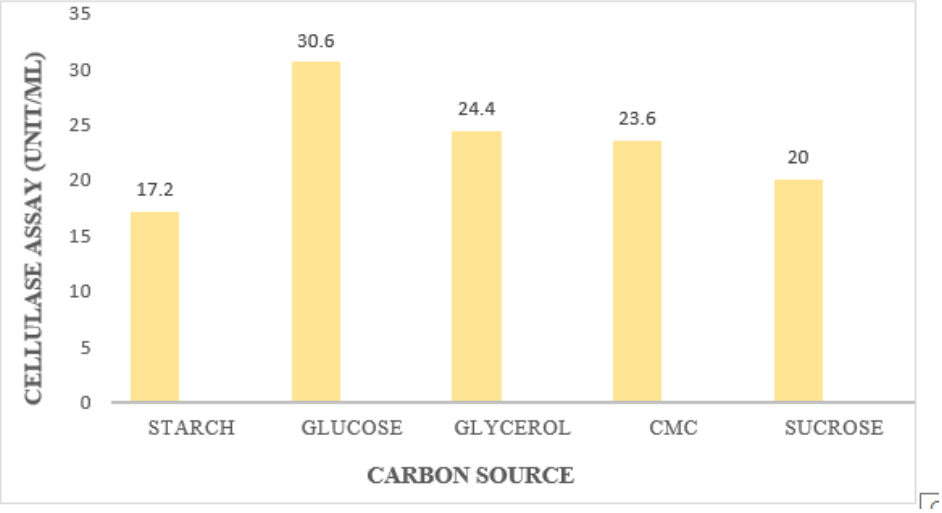

Fig 6: Effect of carbon source of RE11 strain on cellulase production.

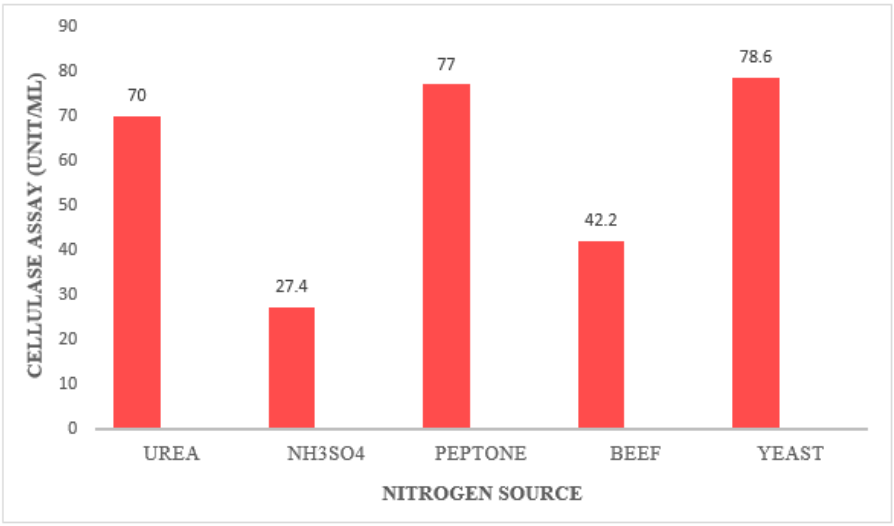

Fig 7: Effect of nitrogen source of RE11 strain on cellulase production

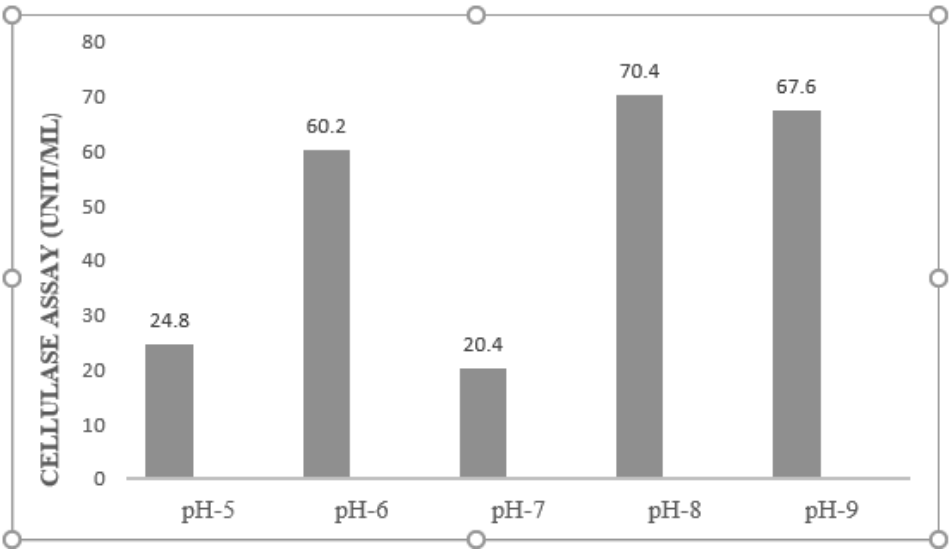

Fig 8: Effect of pH of RE11 strain on cellulase production

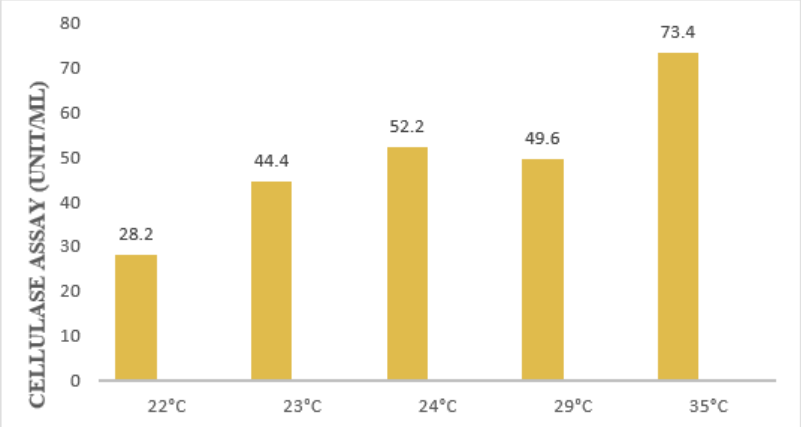

Fig 9: Effect of temperature of RE11 strain on cellulase production

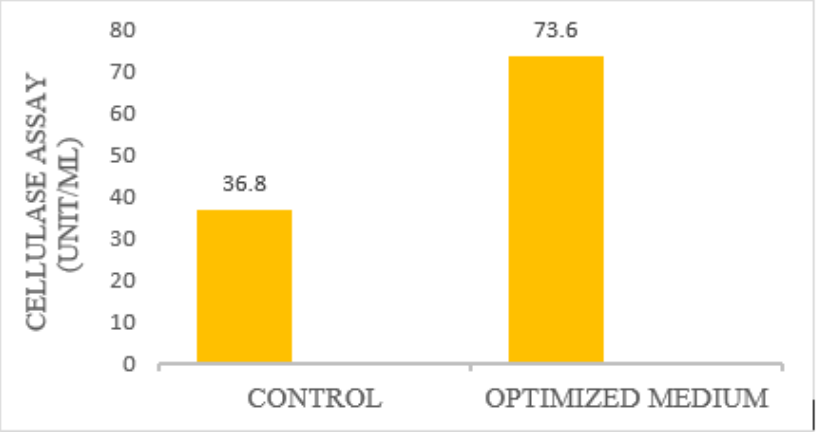

Fig 10: Optimized media of RE11 strain on cellulase production

The biochemical characterization represents positive for Indole, TSI, starch and catalase (Table 2). The molecular characterization of the strain RE11 was done and 16S rRNA sequencing reported the FASTA format of nucleotide sequencing of the characterized RE11 strain. The evolutionary relationship represents the strain *Streptomyces* RE11 has 99 percent similarity with *Streptomyces pactum* (Fig 11). Hence the identified cellulase producing strain was *Streptomyces RE11*. It was submitted under the accession number OR252357.

Table 2: Biochemical Characterization of RE11

S. NO	BIOCHEMICAL TESTS	RESULTS
1	Indole Test	Positive
2	Methyl red Test	Negative
3	Vogues Proskauer Test	Negative
4	Nitrate Test	Negative
5	Citrate utilization	Negative
6	Oxidase Test	Negative
7	Triple Sugar Iron	Positive
8	Urease Test	Negative
9	Starch Test	Positive
10	Catalase Test	Negative

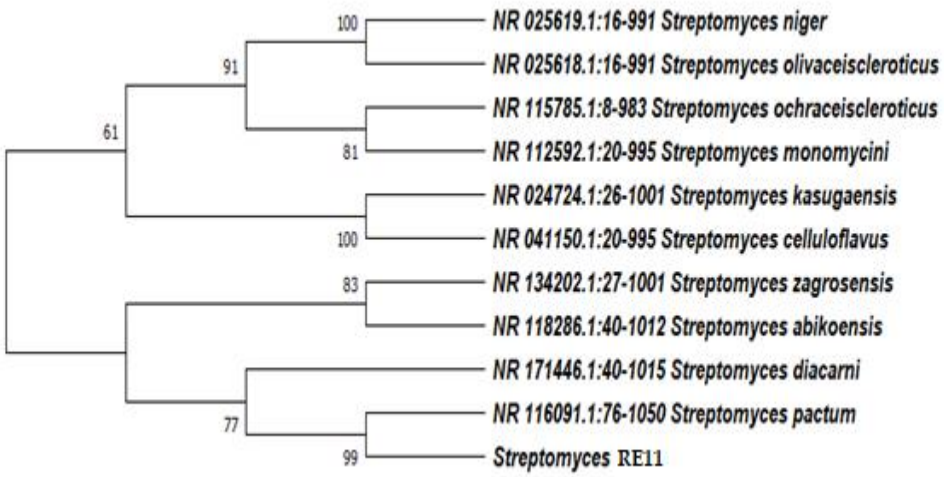

Fig. 11 Evolutionary relationship of the strain *Streptomyces* RE11

Partial Purification Cellulase enzyme from *Streptomyces pactum*:

From the standard assays it can be concluded that the enzyme production was higher after the dialysis of enzyme. The amount of enzyme liberated was found to be 77.6 U/ml. SDS-PAGE analysis of the crude filtrate and precipitated using ammonium sulphate. The two different band was appeared in the SDS-PAGE with different size. The band appeared in the crude protein may be the cellulase enzyme. The determination of enzyme activity gel showed the presence of two different isoforms in the crude enzyme preparation and two bands were seen in the culture filtrate (Fig. 12).

Fig 12: Crude protein and crude enzyme analysis.

Hence it's concluded that the novel strain *Streptomyces* RE11 was isolated from Kalpakkam mangrove Habitat and its effective in the production of the cellulase enzyme with the optimized medium. It was partially purified and further zymogram analysis confirmed.

DISCUSSION:

A study conducted in 2009 by Ramesh S et al. found that 288 marine samples were collected from various locations in the Bay of Bengal, ranging from Pullicat Lake to Kanyakumari. Using starch casein agar medium, 208 isolates of marine actinomycetes were isolated. Documentation of the growth pattern, mycelial coloration, production of diffusible pigment and exo-polysaccharides, and finally the abundance of Streptomyces spp. A significant fraction (88%) of marine actinomycetes were Streptomyces spp. From the 208 isolates, 183, 157, 72 and 68 isolates are capable to produce lipase, caseinase, gelatinase and amylase. From which 116 isolates were produced cellulase enzyme. The Bay of Bengal has a greater chance of producing industrially significant marine actinomycetes due to its diversity, antimicrobial activity, and enzyme production. These organisms may also be essential for the discovery of molecules and/or enzymes that have practical applications in industry [Ramesh S et al., 2009].

In the present study the cellulase production is higher than the finding of [Tabao & Monsalud 2010] who observed 56.60 U/ml, (Bacillus cereus), 66.50 U/ml (Bacillus licheniformis), 50.33 U/ml, (Bacillus spp.), 51.04 U/ml, (Bacillus pumilus), 48.70 U/ml, (Bacillus pumilus) of cellulase activity from Philippines mangrove soil.

Researchers Tawseef Ahmad et al., 2020 [Tawseef et al., 2020] discovered that carbon sources enhance bacterial growth and the synthesis of enzymes. Glycerol, on the other hand, did not increase enzyme activity but did exhibit maximum growth. The maximum enzyme activity in the glucose-containing medium was 17.1 IU/L, which is 12 times higher than in the control. They looked into the different nitrogen sources even more, and in the medium supplemented with ammonium chloride, they found the maximum activity of 1.7 IU/L, or a 1.2-fold increase over the control [Tawseef et al., 2020]. With an activity of 0.028 ± 0.001 U/mL, cellulase was produced optimally at pH 6 and declined slightly (with no significant difference in production) at pH 8–10. The least amount of cellulase was produced at pH 3. Evelyn N. Fatokun, *Uchechukwu* et al., 2016 [Fatokun et al., 2016] looked into how temperature affected the amount of xylanase and cellulase that *S. albidoflavus* strain SAMRC-UFH5 produced. The best temperature for cellulase production was 40 °C, then 35 °C, with activity values of 0.011 ± 0.001 and 0.009 ± 0 U/ml, respectively. We partially purified the enzyme from the novel marine Actinomycetes *Streptomyces* RE11.

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