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CELLULASE ENZYME PRODUCTION FROM RHIZOPHORA MUCRONATA

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ABSTRACT

Mangroves are important habitats seen at tropical and subtropical coastal regions. Endophytes are common in nature. Bacterial endophytes are less studied. In the present study, The *Rhizophora mucronata* sample was collected from coastal area of Thrissur district, Kerala. The bacteria was isolated from the sample by using pour plate technique and the bacteria was tested to produce cellulase by growing them on CMC agar medium for 1 day. It was screened by observing zones around the colonies. The bacteria was further identified using suitable biochemical tests. Then it was inoculated into cellulase production medium. Parameters like pH, temperature, incubation time were optimized. Enzyme activity was measured by DNS method at 540 nm. The results showed that the optimum temperature for cellulase enzyme production was 37°C, pH 8 and incubation period 48 hrs. The enzyme was partially purified by ammonium sulphate precipitation and dialysis. Finally characterized using SDS-PAGE analysis.

KEYWORDS: *CMC agar medium, Endophytic Bacteria, DNS method, SDS- PAGE*

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INTRODUCTION

The demand for industrial enzymes of microbial origin is greatly increased due to their application in a wide range of processes. Cellulase is one of the most useful enzymes in industry. It can be produced by fungi, bacteria or actinomycetes. Bacteria, which has high growth rate as compared to fungi has good potential to be used in cellulase production. Nowadays enormous amount of agricultural and industrial cellulosic wastes have been accumulating in environment. Celluloses are regarded as the most important renewable resource for bioconversion. Many Cellulosic substances were hydrolyzed to simple sugars for making Single Cell Protein, sweeteners etc. It has been become the economic interest to develop an effective method to hydrolyze the cellulosic biomass.[1] Cellulases are the inducible bioactive compounds produced by microorganisms during their growth on Cellulosic matters. Increased knowledge of mode of action of Cellulase; was used in enzymatic hydrolysis of cellulosic substances. Although a large number of microorganisms are capable of degrading cellulose, only a few of them produces significant quantities of cell-free bioactive compounds capable of completely hydrolyzing crystalline cellulose *in vitro.* Numerous investigations have reported the degradation of cellulosic materials, but few studies have examined which microorganisms had met the industrial requirement.The cellulases have attracted considerable attention in recent years due to their great biotechnological and industrial potentials. Cellulose is a polymer of β-1,4 linked glucose with amorphous and crystalline regions. It is hydrolyzed by cellulase, a complex mixture of enzymes containing endo- and exo-glucanases plus cellobiose. The complete hydrolysis of insoluble cellulose requires the synergistic action of these components. Different cellulase preparations vary widely in the proportions of these components, depending on the source, growing conditions of the organism, and harvesting and handling procedures. Cellulolytic enzymes degrade cellulose by cleaving this glycosidic bond. It can be classified into three types: endoglucanases (1,4-b-Dglucan 4-glucohydrolase, EC 3.2.1.4), exoglucanases (b-1,4-D-glucan cellobiohydrolase), and b-glucosidases (b-D-glucoside glucohydrolase, EC 3.2.1.21). Endoglucanases randomly hydrolyze internal b-1,4-glycosidic bonds in Cellulose. To obtain cheap ethanol will depend on the successful screening of novel cellulose producing strain. Since industrial bioconversions of lignocelluloses requires multifunctional cellulase with broader substrate utilization as well as the application of enzymes that can work efficiently in a wide range of temperatures and pH conditions used in the bioconversion of cellulosic material to bioethanol.[2] Cellulases have a wide range of applications, and the main potential applications are in food industry- processing of fruit juices, baking etc., Animal feed preparation- improved digestibility and nutritional quality, Textile industry- used for Biopolishing to increase softness and brightness., Chemical and fuel industry . Other areas of application include the paper and pulp industry- de-inking of paper, Waste management- conversion of renewable cellulosic biomass into commodity chemicals,Medical/ pharmaceutical industry- protoplast production, genetic engineering and pollution treatment. They act synergistically to convert the complex carbohydrates into glucose. Its production from bacteria can be an advantage as the enzyme production rate is normally higher due to the higher bacterial growth rate as compared to fungi. Screening of bacteria, optimization of fermentation conditions and selection of substrates are important for the successful production of cellulase. Bacteria, which has high growth rate as compared to fungi has good potential to be used in cellulase production. Cellulases produced by bacteria are often more effective catalysts. The cellulases have attracted considerable attention in recent years due to their great biotechnological and industrial potentials. They have many industrial applications from the generation of bioethanol , a realistic long-term energy source, to the finishing of textiles, formulation of washing powders, extraction of fruit and vegetable juices, and starch processing.[3] An endophyte is an endosymbiont, often a bacterium or fungus, that lives within a plant for at least part of its life without causing apparent disease. Endophytes are ubiquitous and have been found in all the species of plants. Endophytes promote plant growth and yield, suppress pathogens, and may help to remove contaminants, solubilize phosphate, or contribute assimilable nitrogen to plants[4]. *Bacillus* species produce cellulases: ex; *Bacillus cereus* , *Bacillus licheniformis , Bacillus subtilis , and Bacillus polymyxa. They are known to produce a variety of industrially important enzymes. The gram-positive spore-forming bacteria, Bacillus* strains produced novel cellulases which could liberate glucose from soluble cellulose, carboxymethyl cellulose (CMC), and insoluble crystalline cellulose. Free living cellulolytic bacteria such as *Bacillus sp*. have been studied from the point of understanding the enzyme systems involved in the cellulose degradation. Mangrove plants have adapted to a unique habitat with muddy saline water, anaerobic soil, brackish tidal activities and high microbial and faunal Competition. *Rhizophora mucronata* is commonly known as mangrove growth in the tropical and subtropical region coastlines. It helps to maintain marine life and balances the ecosystem. It is a much branched large shrub or moderate sized tree, up to 10 m tall, supported on adventitious prop roots from stem and branches with reddish brown bark distributed throughout largest mangrove forest. The plant was used in leather industry because of its rich tannin content. The polysaccharides have been reported for anti-HIV activity. Leaves of *Rhizophora mucronata* are poulticed armored fish injuries. Leaf has been used in the folk medicine for treating diarrhea or gastric motility disorder Bark is also mentioned for its anti diarrheal properties. Mangrove has been used as traditional medicine in South Asian countries. Recently scientists are veering in search of effective remedies from mangroves for diseases such as diabetes, asthma, cancer, ulcer, wounds and AIDS. Different communities use it for different purposes such as Indo-Chinese use the roots for angina and hemorrhage, Malayans use old leaves and roots for childbirth, Burmese use the bark for bloody diarrhoea .[5]

MATERIALS AND METHODS

Sample collection

The mangrove plant samples were collected from Thrissur, Kerala.They were stored at 4°C.

Preparation of inoculum

The plant leaf sample was taken and surface sterilized with 70% ethanol and distilled water. One gram of sample was ground with10 ml of sterile distilled water by using clean mortar and pestle. The slurry was filtered by whatman's filter paper and collected in a sterile bottle. This was the inoculum for cellulase enzyme production.[6]

Isolation of endophytic bacteria

Nutrient agar media which contain peptone, beef extract, sodium chloride and agar for bacterial isolation was prepared and sterilized. 1 ml of inoculum was taken and serially diluted. 0.1 ml of serially diluted sample was poured into a Petri plate and evenly distributed to whole of the plate. Above the sample cooled media was poured and kept it for solidification. After the solidification the plates were kept at 37° C for 24 hours and examined for colony formation. The strains were purified by multiple streaking techniques and used for screening of endophytic bacteria for cellulase production.[7]

Screening and Identification of cellulase producing bacteria

The isolated bacteria were tested to produce cellulase by growing them on carboxyl methyl cellulose (CMC) agar medium for 1 day. The plates were then stained with 1% Congo red solution. The cellulase producing bacteria were screened by observing zones around the colonies against the red background. The maximum zone of clearance was selected for further analysis.[8] Various tests like Gram's staining, other biochemical tests like Indole production,Methyl- red test,Voges-proskaver test, Citrate utilization test, Spore staining were carried out using suitable reagents and standard procedures to identify the bacteria. [8]

Production of cellulase enzyme

200 ml of production media was prepared and sterilized, which contains KH_2PO4, K_2HPO4, $MgSO_4.7H2O$, $(NH4)_2SO4$, $CaCl_2.7H2O$, $FeSO_4.7H2O$ and CMC. Then inoculated with 2 ml of inoculums. The flask was kept in incubator at 37°c for 48 hrs.

Optimization

Influence of different temperature for cellulase enzyme production

200 ml of sterile production medium was prepared in different conical flask and inoculated with 2 ml of inoculums. Each flask was incubated at different temperature like 37°C, 45°C and 55°C. The enzyme activity was estimated from the crude enzyme extract.[9]

Influence of different pH for cellulase enzyme production

200 ml of sterile production medium was prepared in different conical flask and inoculated with 2 ml of inoculums. Each flask was incubated at different pH like 5, 6, 7 and 8. The enzyme activity was estimated from the crude enzyme extract.

Influence of different incubation time for cellulase enzyme production

200 ml of sterile production medium was prepared in different conical flask and inoculated with 2 ml of inoculums. Each flask was incubated at different hours,24, 48 and 72. The enzyme activity was estimated from the crude enzyme extract.[10]

Cellulase Assay

Activity of Cellulase enzyme was assayed by using DNS method.10 ml of culture was centrifuged at 5000 rpm for 15 minutes. The cell extract was subjected to enzyme assay. 0.1 ml of enzyme extract was taken with 1 ml of 1% CMC and 1 ml of sodium phosphate buffer were added. The test tubes were incubated at 50°C for 30 minutes. Released reducing sugar was measured by DNS method. After the incubation 3 ml of DNS reagent was added to stop the reaction. This mixture was boiled for 10 minutes in water bath. At the time of cooling 1 ml of freshly prepared sodium potassium tartarate was added and the samples were read at 540 nm in uv spectrophotometer. The enzyme activity was expressed as U/ml. One unit of enzyme activity is defined as the amount of enzyme required to release 1µl of glucose from the substrate per min per ml under standard assay conditions.[11]

Estimation of protein (Lowry's method)

Five ml of alkaline copper reagent was added to the samples and allowed to stand for10 mts. 0.5 ml of folin's phenol reagent was added to it and incubated at room temperature in the dark for 30 mts and OD values for the standard and sample were observed at 660 nm.BSA was used as a standard . The concentration in the sample was estimated from the standard graph.

Partial purification of enzyme

Ammonium sulphate precipitation

The sample was centrifuged in a refrigerated centrifuge at 6000 rpm for 15 minutes at 4°C and the supernatant was collected.

- Known volume of supernatant was placed in a cooling bath on the top of a magnetic stirrer.
- The ammonium sulphate which should be added for different cut offs were reffered from ammonium sulphate precipitation table.
- The ammonium sulphate weighed out for the needed cut off was slowly added.
- Gentle and regular stirring was given. It was continued for 30-60 min after all salt had been added.
- This was spinned at 10000 rpm at 4°C for 20 min.
- The pellet was resuspended in 1-2 volume of buffer and ammonium sulphate can be removed by dialysis.[10]

Dialysis

Dialysis is a process of removal of small molecules from a sample containing mixture of both large and small molecules. Semi permeable membrane can be used for dialysis.

SDS page

Thoroughly clean dried plates and spacers were assembled properly. It was held together with clips, clamped in an appropriate position. White petroleum jelly was applied around the edges of the spacer to hold them in a place and sealed with chamber between the glass plates. Sufficient volume of separating gel mixture was prepared and poured in the chamber between the glass plates. Left to set for 40-60 mts. The stacking solution was poured above the separating gel and the comb was placed in the stacking gel was allowed to set for 30 – 60 mts. After polymerized the gel, comb was removed without disturbing the gel. The gel was installed after removing the clips in electrophoretic apparatus. It was filled with running buffer and any trapped bubbles at the bottom of the gel were removed. The cathode was connected at the top and turn on the power.The samples were prepared for electrophoresis. 15µl of sample was mixed with 15 µl of sample buffer. The mixture was loaded into the well. A well was loaded with standard protein marker in the sample buffer. Turn on the current to 10-15 MA for initial 10- 15 mts until the samples travelled through the stacking gel. Their run was continued at 30 A until the bromophenol blue reaches the bottom. After the run was complete, the gel was removed carefully from the plates and immersed in the staining solution for 3 hours. After that the gel was transferred into destaining solution and shaken gently. Dye that was not bound to protein was removed. The destainers was changed frequently until the background of gel is colourless. The proteins fractionated into bands were seen blue color.[12]

RESULTS AND DISCUSSION

The bacteria were isolated from *Rhizophora mucronata* by pour plate method. The identification method was followed to detect the isolated organism. From the preliminary test of gram staining a purple color rod shaped organism was observed. This indicates the organism is gram positive and this organism showed positive result for endospore staining with green color spore inside pink color cell. For the confirmation of organism Biochemical tests were followed;

Table 1
Biochemical tests and the results.

TESTS	RESULTS
Indole test	-ve
Methyl Red	-ve
Voges-Proskaur	+ve
Citrate test	+ve

These results showed that the organism is *Bacillus*.The *Bacillus* strain was confirmed for cellulase production by the formation of clear zone around the strain in CMC agar plates.

Enzyme activity

The CMC media was inoculated with bacteria and kept it for incubation in a shaker. enzymatic assay was performed.

pH

From the results, (table 2) it was observed that the optimum pH for cellulase enzyme production was pH 8.

Temperature

From the results (table 3), it was observed that the optimum temperature for cellulase enzyme production was 37°C.

Incubation period

From the results (table 4), it was observed that the optimum incubation period for cellulase enzyme production was 48 hrs.

Table 2
Effect of different pH on enzyme activity

pH	ENZYME ACTIVITY (IU/ml)	SPECIFIC ACTIVITY (IU/mg)
	26.66	18.62
6	43.02	20.83
7	50.01	22.67
8	55.30	24.92

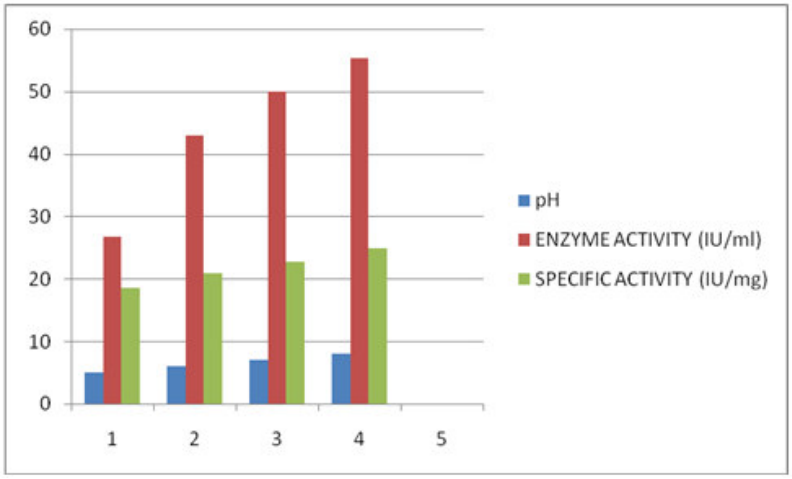
Table 3
Effect of different temperature on enzyme activity

Temperature(°C)	ENZYME ACTIVITY (IU/ml)	SPECIFIC ACTIVITY (IU/mg)
37	40.30	32.40
45	37.19	28.35
55	28.66	14.58

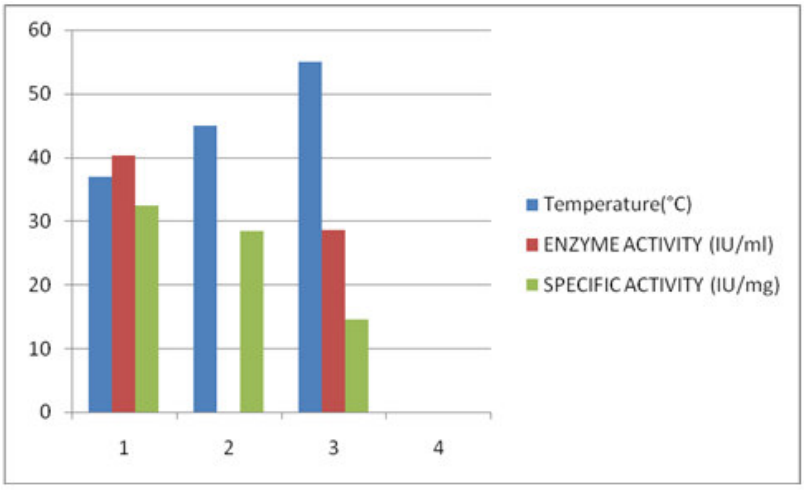
Table 4
Effect of different incubation time on enzyme activity

INCUBATION TIME (hrs)	ENZYME ACTIVITY (IU/ml)	SPECIFIC ACTIVITY (IU/mg)
24	53.38	10.66
48	60.22	25.46
72	45.67	15.33

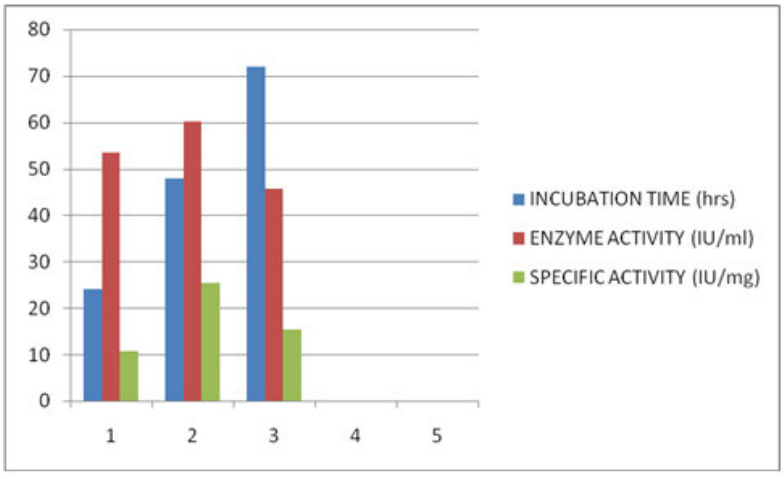
Graph 1
Effect of different pH on enzyme activity



Graph 2
Effect of different temperature on enzyme activity.



Graph 3
Effect of different incubation time



SDS page

The cellulase enzyme was isolated from *Bacillus.* Then dialysis was done and the dialysate enzyme was subjected to SDS Page for separating protein .15µg of samples and marker was loaded in the wells. The protein was observed in the well but the result showed that shearing pattern of band which are separated so further standardization methods are (volts, volume of sample, staining,) needed for separation.

Figure 1
Isolated cellulase producing bacteria

Figure 2
Production media

Figure 3
Dialysis

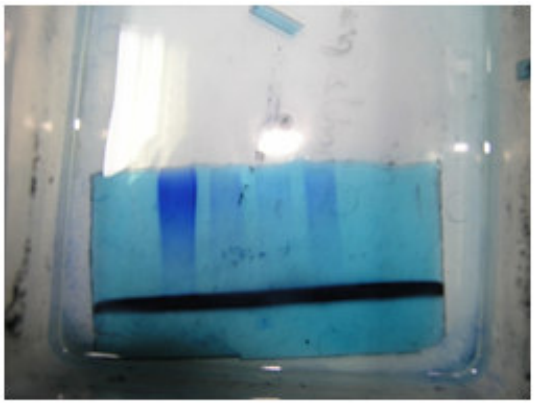


Figure 4
SDS-page gel

DISCUSSION

The *Rhizophora mucronata* sample was collected from coastal area of Thrissur district, Kerala. The bacteria were isolated from the sample by using pour plate technique and the bacteria were tested to produce cellulase by growing them on CMC agar medium for 1 day. It was screened by observing zones around the colonies. Then it was inoculated into cellulase production medium. Different parameters like pH, temperature, incubation time were optimized. Enzyme activity was measured by DNS method at 540 nm. The result showed that the optimum temperature for cellulase enzyme production was 37°C, pH - 8 and incubation period - 48 hrs. The enzyme was partially purified by ammonium sulphate precipitation and dialysis. Finally characterized using SDS-PAGE analysis Cellulase can be easily produced and is one of the most useful enzymes in industry. They have a wide range of applications, and the main potential applications are in food, animal feed, textile, fuel, and chemical industries. Other areas of application include the paper and pulp industry, waste management, medical/pharmaceutical industry, protoplast production, genetic engineering, and pollution treatment.

CONCLUSION

At present, cellulases and related enzymes are used in food, brewery and wine, animal feed, textile and laundry, pulp and paper industries, as well as in agriculture and for research purposes.Therefore there is always an increase in demand for the enzyme. The present study is aimed at isolating the endophytic microorganism capable of producing the enzyme and purification of it. The enzyme is found to have maximum activity at a temprature of 37°C and a pH of 8. The enzyme thus performs well under normal conditions of temprature and pressure. This enzyme can be thus checked for its utilization in various industries.The enzyme production using *Bacillus* sp. was found to be very high in short duration of time. The produced cellulase enzyme is easily degradable and ecofriendly. The bacterial endophytes are less studied and the cellulase enzyme produced by the endophytes of the mangroves can be further purified and analyzed for their use in different industries. The enzyme production can further be enhanced using suitable techniques so that it is proved cost effective benefitting not only the human kind but also leading to the true protection of the nature.

CONFLICT OF INTEREST

Conflict of interest declared none.

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