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Isolation and Identification of Cellulase Producing Bacteria from Gut Microbiota of *Coptotermes ceylonicus* (Termite)

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Abstract:
Cellulose and lignocellulose, are abundantly available biomass, derived from plant sources. Apart from this, lignocellulose, is also known to be the main component of municipal solid waste, which needs bioremediation. Because of their bioethanol production capabilities, these polysaccharides, may also be used as renewable energy sources, replacing the fossil fuels. Biodegradation of cellulose and lignocelluloses to simpler sugars and ethanol production may be processed by an important enzyme, cellulase. This enzyme, occurs naturally in the gut of isopteran insects, termites. The gut microbiota, of these white ants synthesize cellulase and are capable of digesting cellulose and lignocelluose. Cellulases derived from microbes of termites, are considered to be natural and cost effective, enzyme reserves. In this context, the present study, was attempted to extract cellulase from gut bacteria of termites. White ants, were collected from wood, and their gut microbes, were isolated, identified and the cellulase was extracted. The bacterial species which was isolated in the present study was identified as *Pseudomonas aeruginosa.* Gram's staining and different biochemical tests, were performed, to ascertain its identity. The cellulase enzyme, which was extracted was partially purified, by the method of ammonium sulphate precipitation. This study, reiterates, that termites, may be considered as the best economical source for cellulase extraction, which can be used for degradation of cellulose and lignocelluloses, and also for bioethanol production.

Keywords: *Pseudomonas aeruginosa*, Termites, Cellulase, Cellulose, Lignocellulose Bioethanol, Biodegradation.

INTRODUCTION

Cellulose is a renewable source of energy and hence cellulose hydrolysis a subject of intense research and industrial interest (Bhatt, 2000). In addition to cellulose, lignocellulosic biomass (LB) also has emerged as a promising alternative to fossil fuels (Pang *et al.,* 2010). Lignocellulosic materials which are available abundant in the biosphere can be turned into a worthwhile material, but sadly they are burnt off for disposal throughout the world. (Levine, 1996).Municipal solid waste is also rich in lignocellulosic material mainly composed of cellulose and lignin with a small ratio of hemicellulose (Malherbe and Cloete, 2003). Lignocellulosic materials can be converted into valuable substances such as different organic acids and antibiotics (Banjo and Kuboye, 2000). Studies have shown that

lignocelluloses can be degraded by different strains of bacterial species, such as *Cellulomonas, Pseudomonas* and *Actinomycetes* species which hydrolyze lignin as well as the carbohydrate portion of the waste (Doolotkeldieva and Bohusheva, 2011). Similarly cellulose, is also degraded by microorganisms such as bacteria, fungi and actinomycetes, because they release cellulase enzyme. Cellulase enzyme, from the bacteria, apart from playing an important role, in degradation of cellulose and lignocellulose, has an important application in many industries. Bacterial cellulases are frequently used as highly effective and potent catalysts (Gautam, 2010). Similarly, *Actinomycetes* from the genera *Streptomyces* and *Thermobifa* and other genera of bacteria, such as *Pseudomonas* and *Sphingomonas* are some of the sources of enzymes used for decolorization and degradation of textile dyes (McMullan, 2001). In paper and pulp industry, cellulases are used in pulping, bioremediation of industrial waste, bleaching and fiber enhancement. The application of cellulases in agriculture is reported in enhancement of crop growth and also as a control agent of plant diseases. Cellulases are also used as feed additives for significant improvement in feed conversion ratio as well as digestibility of the cereal - based foods (Baker and Wicker 1996). These studies, clearly indicate the biological and industrial importance of cellulase enzyme, and therefore, its extraction from a more economical and natural source would be more appealing rather than obtaining it from a synthetic source. In this context, the economic value of small isopteran insects, known as termites, may be recalled, as they are capable of degrading cellulose. Termites, miniature white ants, are often found in the soil, and are known to decompose, cellulose and lignocellulose, with the aid of gut microbial flora which they harbor. They are known to harbor a dense and diverse array of prokaryotes (Ohkuma, 2003), which are known to be the source of cellulase enzyme, and therefore, in the present study, we attempted to extract cellulase enzyme, by isolating and identifying the gut micro flora, from the termites.

MATERIALS AND METHOD

Termite collection and isolation

Termites were collected from a wood shop in Ayanavaram which is located in Chennai. Termites were surface sterilized using 70% ethanol for 30 seconds and the whole bodies of termites were macerated using mortar and pestle. The paste obtained from the termite was used for isolation of the bacteria. The bacteria was inoculated on the media by swabbing technique

Screening of Cellulase Producing Organisms

Nutrient broth agar media was used for the inoculation of bacteria. Then for primary screening of cellulase producing bacteria, the bacteria from the colonies of nutrient broth agar plates were inoculated in the Carboxymethyl cellulose media as it preferably supports the growth of cellulolytic bacteria and pure culture was obtained by using the preparation of same media. The secondary screening of cellulase producing organisms was done by congo red test.

Agar plates were prepared with 1% CMC strains were streaked and petri plates were incubated at 37˚C for 48 hours. Petri plates were flooded with 0.1% Congo red reagent and left for 20 minutes. Then the plates were washed with 1M NaCl. Clearance zones called halo zones are seen against the red color of Congo red for the positive test. Enzyme activity was indexed as.

$$Cellulolytic\ Index = \frac{Diameter\ of\ zone - Diameter\ of\ bacterial\ colony}{Diameter\ of\ bacterial\ colony}$$

And only the isolates that produced a clear zone around the colony were chosen for further study.

Identification of Cellulolytic Bacteria

Identification of cellulolytic bacteria was carried out, which was based on morphological and biochemical tests using Bergey's Manual of Determinative Bacteriology 5th edition.

Morphological Characterization (Gram's Staining)

The smear was prepared by using bacteria from colony mixed with a drop of distilled water in a clean slide. The slide was subjected to heat fixation. The slide was flood with crystal violet for 2 minutes then washed with distilled water. Gram iodine were flooded for 2 minutes washed with ethanol and

then with distilled water.Finally, flooded with safranin for 2 minutes washed with distilled water and air dried.Then the slide is subjected to microscopic analysis.

Biochemical Analysis

Indole Test (Trytophanase Hydrolysis): This test is performed to determine the ability of an organism to produce indole from the aminoacid tryptophanase. The isolates were incubated in the broth at optimum temperature for 24- 28 hours. Kovac's reagent (10 – 12 drops) was added to broth and observed for the formation of colored layer on the surface of the medium.

Methyl Red Test (MR Test): This test was performed to determine the ability of an organism to produce mixed acid and products from glucose fermentation. The isolated were inoculated into MR – VP broth and incubated for 3-5 days at optimum temperature. Three to four drops of methyl red reagent was added to interpret the MR positive and MR negative isolates, based on red or yellow color development.

Voges – Proskaur Test (VP Test): This test was executed to determine the ability of an organism to produce action, 2, 3 butanediol; and ethanol. The isolates were inoculated into MR – VP broth and incubated for 3-5 days to optimum temperature. One milliliter of culture was pipette out and to it Barritt's solution A (alpha – haphthol) and Barritt's solution B (KOH) were added in equal proportions. The solution was agitated vigorously and incubated for 15 to 20 minutes for the formation of red color.

Citrate Utilization Test: This test was performed to determine whether the organism is capable of using citrate as the sole source of Carbon with production of the enzyme citritase. The isolates were streaked onto Simmons citrate agar slants and incubated at optimum temperature for 24 - 48 hours and observed for any color change.

Triple Sugar Iron Agar Test (TSI Test): The ability of an isolate to produce H_2S (Hydrogen sulfide) was carried out by stabbing the isolate into TSI (Triple sugar Iron Agar) medium and incubated. Gaps, cracks or bubbles in the agar medium indicate the gas production.

Cellulolytic Activity Determination

Enzyme Production: Production medium contained (g/100ml) glucose 0.05 gram peptone 0.075 gram, FeSO4 0.001 gram, KH2PO4 0.05gram and MgSO4 0.05 gram. Ten millilitres of medium were taken in a 100ml conical flask. The flasks were sterilized in autoclave, and after cooling, the flask was inoculated with overnight growth bacterial culture. The inoculated medium was incubated at 37˚C in shaker incubator for 24 hours. At the end of the fermentation period, the culture medium was centrifuged at 5000 rpm for 15 minutes to obtain the crude enzyme, which served as enzyme source.

Enzyme Assay: Cellulase activity was measured following the method of Miller (1959). Briefly, a reaction mixture composed of 0.2 ml of Crude enzyme solution plus 1.8 ml of 0.5% carboxymethyl cellulose (CMC) in 50mM sodium phosphate buffer (pH7) was incubated at 37˚C in a shaking water bath for 30 minutes. The reaction was terminated by adding 3ml of DNS reagent. The color was then developed by boiling the mixture for 5 minutes. OD of samples was measured at 575 nm against a blank containing all the reagents minus the crude enzyme.

Purification of Cellulase

Ammonium Sulfate Precipitation: The prepared crude enzyme was saturated with solid ammonium sulphate at different concentration of 40, 60, and 80 grams. The mixture was kept overnight at 4˚C in a magnetic stirrer. Then the mixture was centrifuged and the pellet was dissolved in 50 mM Sodium Phosphate buffer saline at pH 7.0 for further purification. The partially purified sample was assayed for enzyme activity by using DNS method.

RESULTS

The results are tabulated and discussed below. Termites were collected and the gut content was analysed for the identification of cellulase producing bacteria.

Screening of cellulase Producing Organisms

The macerated pastes of termites were streaked on the nutrient agar media resulting in the formation of 15 – 20 bacterial colonies (Plate – 1). The bacteria from those colonies inoculated on CMC media to obtain Pure culture (Plate – 2). Out of which one isolate were found to be potential cellulase degrading bacteria which was subjected to Congo red test. Clearance zone called halo zone are seen against the red colour of Congo red and the test resulted positive with the cellulolytic index of 3.25 (Plate – 3).

Morphological Characterization

Gram Staining: Morphological characters were determined by Gram's staining technique. Gram's staining microscopic analysis showed that the isolated bacteria are Gram negative rod shape (Plate – 4).

Biochemical Analysis

A series of biochemical tests such as Indole Test, Methyl Red Test (MR Test), Voges Proskaur Test (VP Test) Citrate Utilization Test, Triple Sugar Iron Agar Test (Tsi) were conducted for the purpose of identification of bacteria (Table – 1).

Identification of the Isolate

The morphological characters analysed by Gram's staining and different biochemical tests showed that the isolated bacteria was identified as *Pseudomonas aeruginosa* with reference to Bergery's manual.

Enzyme Assay of Crude Enzyme

The OD of the crude cellulase was measured at 575nm against the blank showing 0.1498IU / ml (Table – 2).

Purification of Cellulase

Ammoniun Sulphate Precipitation: The partially purified sample using ammonium sulphate was assayed for enzyme activity by using DNS method showed 0.1383IU / ml of activity (Table – 3).

Table1: Biochemical Analysis

S. No.	TEST	RESULTS
1.	Indole Test	+
2.	Methyl Red Test (MR Test)	_
3.	Voges Proskaur Test (VP test)	_
4.	Citrate Utilization Test	+
5.	Triple Sugar Iron Test (TSI)	+

Table 2: Enzyme Assay

S. No.	Cellulase Sample	Enzyme Activity Iu/Ml
1.	Blank	0
2.	Crude Enzyme	0.1498

Table 3: Partial Purification of Cellulase

S. No.	Cellulase Samples	Enzyme Activity Iu/Ml
1.	Crude Enzyme	0.1498
2.	Partial Purification	0.1383

Table 4: Significance of Enzyme Activity between Crude and Partially Purified Enzyme

S. No.	Samples	Enzyme Activitylu/Ml	P Value
1.	Crude Enzyme	0.1498	0.004686
2.	Partially PurifiedEnzyme	0.1383	

Plate 1: Bacterial Colony on Streaking the Nutrient Agar Media

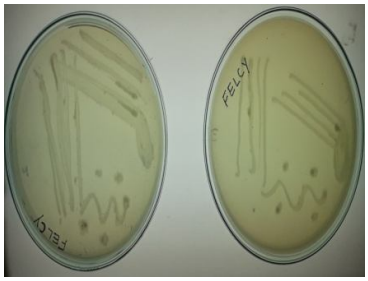

Plate 2: Pure Culture Plates

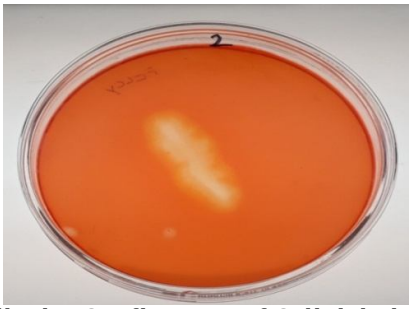

Plate 3: Congo red Test for Qualitative Confirmatory of Cellulolytic Activity

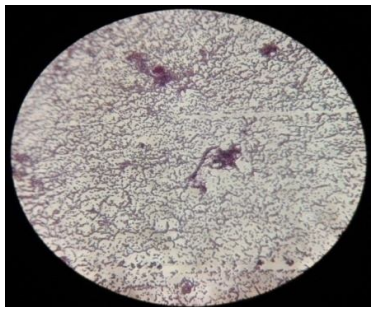

Plate 4: Gram's staining

DISCUSSION

In the present study, we successfully isolated and identified, the bacterial species, from the microbiota ofthe gut of the termites. Based upon the morphological characterization and biochemical analysis the species was identified, as *Pseudomonas aeruginosa.* The gut bacteria, produced cellulase enzyme, which was partially, purified using ammonium sulphate precipitation. These results coincide with that of Subodh K. Upadhyaya *et al.,* (2012) who have also isolated and characterized the cellulolytic bacteria from the gut of termites. Degradation of cellulosic materials is a complex process requiring participation by a number of microbial enzymes, and this can be easily achieved by gut microbiota of termites, which can easily degrade cellulose and lignocellulose (Brune, 2014 and Manjula *et al*., 2014). As discussed earlier, cellulase enzyme, find its application in a vast number of manufacturing entities, such textile industries, paper and pulp manufacturing units, and also in the processing of antibiotics and other organic drugs. Utilization of cellulase enzyme, is not just limited to these industries, but also plays an important role in bioethanol production. The most important and valuable attribute of cellulase, is its capability of degrading the lignocelluloses (considered as waste) and efficiently converting it into ethanol, which may be used as biofuel. This high degree of efficiency is because of about 200 different species of microbes that inhabit termite hindguts, which help in breaking down of complex lignocellulose polymers within the wood into simple sugars by fermenting bacteria and using enzymes that produce hydrogen as a by-product. From these studies, it is quite evident that cellulase enzyme, is an important enzyme, and its, extraction from a more natural source, such as termites is a wise economically viable option, in contrast to artificial synthesis. Thus, the present study reveals that the social worker termites are one of the important insects for isolating potential microorganisms producing novel cellulase enzyme.

CONCLUSION

Termites are more promising arthropod for isolation of potential microorganisms producing lignocellulose degrading enzymes. Biowaste from industries forms the habitat for these potential microbes and provide substrate for cellulase production. In this way it accomplishes two purposes as waste disposal management as well as providing source for enzyme production.

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