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Optimization of Conditions and Production of Carboxy Methyl Cellulase by Bacteria Isolated from Higher Termite Soil

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Abstract

The current work deals with the studies of isolation and preliminary characterization of the bacteria isolated from higher termite soil. Termites play an important role in the turnover and mineralization of complex biopolymers, such as wood and other cellulose and hemicelluloses containing materials. Lignocellulose is the most predominant component of the woody and dead plant materials, as well as it is the most abundant biomass on earth, especially in terrestrial ecosystems. Thus the work focuses on using lingo cellulosic waste efficiently for production of Carboxy Methyl Cellulase (CMC). As CMCase have higher hydrolyzing capacity than the other two cellulases, cellobiase and cellobiohydralases it has wide application in industry such as bread and baking industry, laundry etc. Thus the work focuses on identification of efficient bacterial species in this work 6 bacterial species were isolated among which I4 produced higher CMCase enzyme. After the morphological, biochemical and identification test, the I4 isolate was found to be *Bacillus* sp.

Keywords: Carboxy methyl cellulase (CMC); Cellulase

Introduction

Termites are a ubiquitous feature of tropical and subtropical soils, where their number exceeds 6000 m^{-2} and their biomass densities (>50 gm^{-2}) often surpass those of grazing mammalian herbivores (0.0-7.5 gm^{-2}). Both higher and lower termites have microbes and enzymes in their hindgut, and this is therefore where the most symbiosis occurs. Soil on the other hand is a highly heterogeneous environment [1] that contains a high diversity of microorganisms [2].

These microorganisms influence above ground ecosystems by contributing to soil structure and fertility among other roles [3]. Soil microorganisms are a valuable source of natural products providing important antibiotics for pharmaceuticals, enzymes and bioactive compounds for industries [4]. Since soil being a good habitat for the growth of many number of microorganisms, majorly observed microorganisms are bacteria: *Bacillus* sp, *Klebsiella* sp, *Pseudomonas* sp, *Serratia* sp, *Xanthomonas* sp etc. Many fungal species are also obtained from higher termite soil, which include *Aspergillus* sp, *Phoma* sp, *Neurospora* sp, *Trichoderma* sp, *Penicillium* sp. The major actinomycetes species observed are *Streptomyces* sp, *Geosmin* sp, *Nocardia* sp. Cellulase consists of three different types of enzymes named as endoglucanases, exoglucanses and cellobiases. Several novel enzymes capable of degrading cellulose into sugars have insights from this discovery to create a high performance enzyme cocktail for processing plant biomass into biofuel.

Two cellulases, endoglucanase (CMCase), exoglucanases or β-1,4 glucan hydrolases enzymes were first reported to exist in termitomyces conidiophores of the "fungus garden" Bacterial cellulases have proved to be a better candidate than other microbial cellulases, with their secreted free cellulose complexes comprising all three components of cellulose. Plant cell walls are the most abundant renewable sources of fermentable sugars on the earth [5] and are the major reservoir of fixed carbon in nature. The major components of plant cell walls are cellulose, hemicellulose and lignin, with cellulose being the most abundant component. But plant woods commercially used for the production of paper. So an alternative substrate is required for production of cellulase enzyme. Rice straw, wheat straw, rice bran, wheat bran, when used as substrate there are some bacterial species mainly considered pathogen and degrades these substrates easily.

Several studies were carried out to produce cellulolytic enzymes from bio waste degradation process by many microorganisms including fungi such as *Trichoderma, Penicillium, Aspergillus* spp. etc. by Mandels M et al., Hoffman RM et al., Brown JA et al., Lakshmi Kant et al. [6-9] etc. Similarly cellulolytic property of bacterial species like *Pseudomonas, Cellulomonas, Bacillus, Micrococcus and Cellovibrio* sp were also reported. The specific cellulolytic activity shown by the bacterial species is found to be depending on the source of occurrence [10]. Some features of natural cellulosic materials are known to inhabit their degradation or bioconversion [11,12]. These are degree of crystallinity, lignification and the capillary structure of cellulose to cellulolytic enzymes and other hydrolytic agents. However, many physical, chemical and microbial pre-treatment methods for enhancing bioconversion of cellulosic materials have been reported [12,13]. Since the production of cellulase enzyme is a major process and economically viable, much work has been done on the production of cellulase from lignocellulosics and major attention has been given to use bagasse as substrate [12,13]. The bioconversion of various complex cellulosic waste materials such as bagasse [13], corncob [14]; saw dust [12] have been reported (Figure 1).

Materials and Methods

Sample collection

The higher termite soil sample was collected from three different regions of Palakkad district (Nelliyampati, Kallepully, Chittur). The samples were collected, serially diluted and was spread plated.

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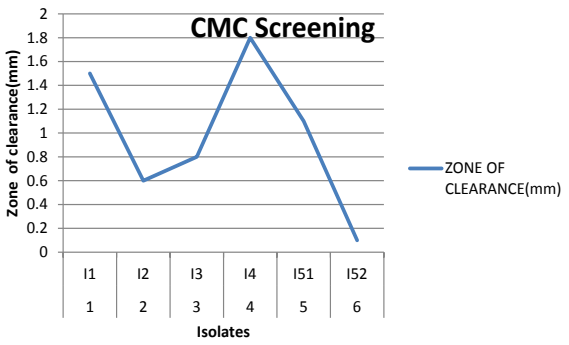

Figure 1: Preliminary CMC screening.

Isolation of bacteria from higher termite soil

The soil samples were isolated and were spread plated from the three soil samples. The six Colonies with visually distinguishable morphologies were selected and restreaked on Nutrient agar to obtain pure cultures isolates were labeled as I1, I2, I3, I4, $I5_1$, $I5_2$ (Tables 1 and 2).

Identification of bacteria

Bacteria were identified and classified based on their physical and biochemical characteristics. Various biochemical test such as IMViC, TSI, Carbohydrate fermentation, Catalase, oxidase test, starch hydrolysis test were performed to identify the bacterial species (Tables 3-5).

Screening

The six isolates were inoculated by spot inoculation and incubated at 24-48 hrs.1% of Congo red solution was added to the spot inoculated plates and then excess stains were removed by using 1M NaCl and zone of clearance were observed as in Figure 1.

Optimization of physiological condition

Cellulases have versatile applications in textile, laundry, pulp and paper, fruit juice extraction, and animal feed additives [15]. In addition, they find use in saccharification of lignocellulosic agro residues to fermentable sugars which can be used for production of bioethanol, lactic acid, and single-cell protein [16]. Bacteria have been widely explored for cellulase production owing to their high growth rate, expression of multi enzyme complexes, stability at extreme temperature and pH, lesser feedback inhibition, and ability to withstand variety of environmental stress [15]. Among them, *Bacillus* sp. continues to be dominant bacterial workhorse due to the capacity to produce and secrete large quantities of extracellular enzymes [17]. However, physical process parameters such as temperature, pH, and agitation speed play a vital role for the cellulase production efficiency of the microorganisms. Agitation speed is an important factor which governs the dissolved oxygen level in the culture broth that affects cell growth of cellulase producing microorganism [18].

Effect of temperature

For estimation of optimum temperature, the enzyme activity was carried out at five different temperatures (20°C, 30°C, 40°C, 50°C and 60°C) using 0.5 ml of Carboxymethyl cellulose substrate.

Effect of pH

The best pH for enzyme activity was determined by enzyme assay at different pH levels (4, 5, 6, 7, 8). In order to find the effect of pH also 0.5 ml of carboxymethyl cellulose is used as substrate.

Results and Discussion

The three samples were collected from higher termite soil. Serially diluted higher termite soil sample showed distinct colonies at 10^{-4} dilution. The selected colonies were labeled as I1, I2, I3, I4, $I5_1$, $I5_2$ and was subjected to physical and biochemical characterization, the results were tabulated in Tables 1 and 2.

The Figure 1 shows the specific screening and CMCase assays. After the preliminary screening the I4 isolate was found to be more efficient for the CMCase activity. Optimization of physiological conditions (pH and temperature) were analyzed as in Figures 2-5, thus I4 isolate at temperature 40°C at pH 6 was more efficient as in Figures 6-9. In addition there were reports that the cellulase production by *Aspergillus niger* and bacterial strains such as *Cellulomonas* sp was observed over a wide range of temperatures between 30 to 50°C [19,20]. In the current research since the I4 isolate utilized the Carboxy methyl cellulose during the screening test. The isolates was further analysed for the optimization: Temperature and pH.

Serial dilution	Colonies obtained (one quadrant)	Colonies obtained (four quadrant)
10-1	525	2100
10-2	326	1304
10-3	245	980
10-4	**200**	**800**
10-5	163	652
10-6	113	452
10-7	98	392

Table 1: Enumeration of bacterial colonies.

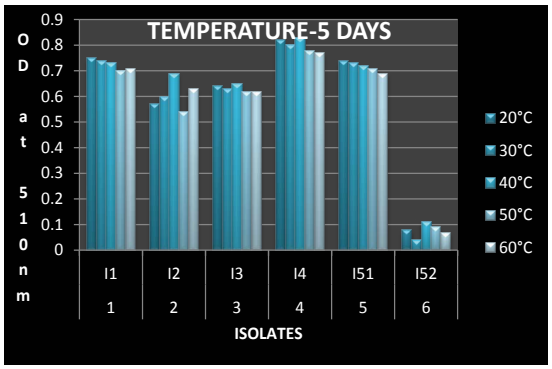

Figure 2: For estimation of optimum temperature, the enzyme activity was carried out at five different temperatures (20°C, 30°C, 40°C, 50°C and 60°C) using 0.5 ml of Carboxymethyl cellulose substrate for 5 days.

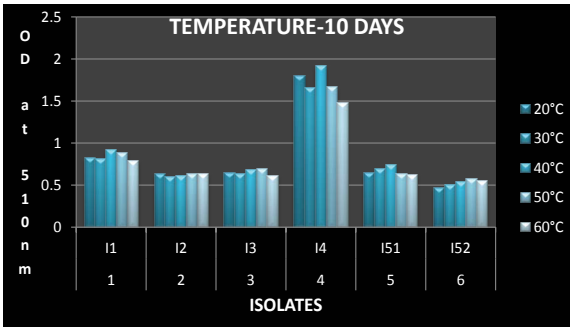

Figure 3: For estimation of optimum temperature, the enzyme activity was carried out at five different temperatures (20°C, 30°C, 40°C, 50°C and 60°C) using 0.5 ml of Carboxymethyl cellulose substrate for 10 days.

S No	Name	Size	Shape	Colour	Margin	Surface	Elevation	Transparency	Viscosity
1.	I1	Large	Undulate	Yellow	Undulate	Smooth	Flat	Opaque	Moist
2.	I2	Medium	Circular	Light yellow	Entire	Smooth	Flat in growing	Transparent	Moist
3.	I3	Small	Circular	Light white	Entire	Smooth	Low convex	Translucent	Dry
4.	I4	Large	Circular	Creamy	Entire	Smooth	Flat in growing	Opaque	Moist
5.	$I5_1$	Small	Circular	White	Diffuse	Finely granular	Flat	Opaque	Ropy
6.	$I5_2$	Small	Irregular	White	Diffuse	Glossy	Flat	Opaque	Mucoidal

Table 2: Physical Characterization.

S No	Bacterial isolates	Gram stain	Microscopic features	Indole test	Methyl red	VP test	Citrate utilization	Oxidase	Catalase
1.	Isolate 1	-ve	Rod shaped	-ve	-ve	+ve	+ve	+ve	+ve
2.	Isolate 2	-ve	Rod shaped	-ve	+ve	+ve	+ve	+ve	+ve
3.	Isolate 3	-ve	Rod shaped	-ve	+ve	+ve	+ve	+ve	-ve
4.	Isolate 4	+ve	Rod shaped	-ve	+ve	+ve	-ve	+ve	-ve
5.	Isolate 5_1	-ve	Rod shaped	-ve	+ve	-ve	+ve	+ve	+ve
6.	Isolate 5_2	-ve	Rod shaped	-ve	+ve	+ve	+ve	+ve	+ve

Table 3: Microscopic Examination and Biochemical Characterization.

S No	Isolates	Sucrose	Glucose	Mannitol	Inositol
1.	I1	Ap/NG	Map/NG	Map/NG	Map/NG
2.	I2	Ap/NG	Map/NG	Map/NG	Ap
3.	I3	Ap/NG	Map/NG	Map/NG	Map/NG
4.	I4	Ap/NG	Nc	Ap/NG	Map/NG
5.	I51	Map/NG	Map/NG	Map/NG	Map/NG
6.	I52	Ap/NG	Map/NG	Map/NG	Map/NG

Abbreviations: Ap/NG: Acid production/No gas; NG: No gas; Map: Minor acid production; Ap: Acid production; Nc: No change

Table 4: Carbohydrate Fermentation Test.

S No	Isolate	TSI
1.	I1	(AK_S/A_B)
2.	I2	(AK_S/A_B)
3.	I3	(AK_S/A_B)
4.	I4	A_SA_B
5.	I51	Nc
6.	I52	(AK_S/A_B)

Abbreviations: AK_S/A_B: Alkaline slant/acid butt; N_c: No change; A_s/A_B: Acid slant/ Acid butt

Table 5: Triple sugar iron test.

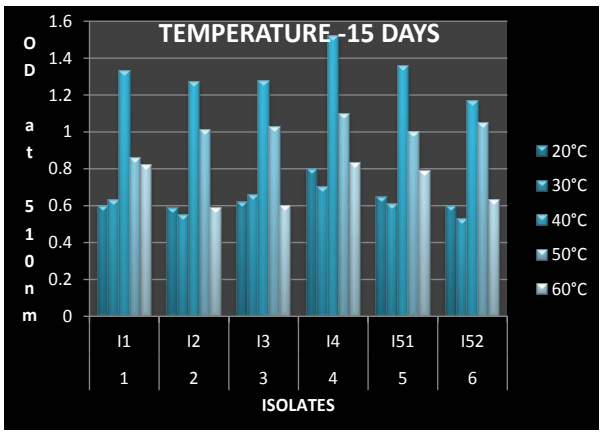

Figure 4: For estimation of optimum temperature, the enzyme activity was carried out at five different temperatures (20°C, 30°C, 40°C, 50°C and 60°C) using 0.5 ml of Carboxymethyl cellulose substrate for 15 days.

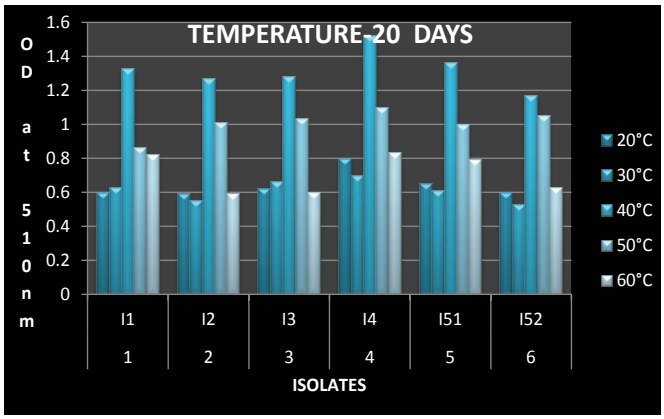

Figure 5: For estimation of optimum temperature, the enzyme activity was carried out at five different temperatures (20°C, 30°C, 40°C, 50°C and 60°C) using 0.5 ml of Carboxymethyl cellulose substrate for 20 days.

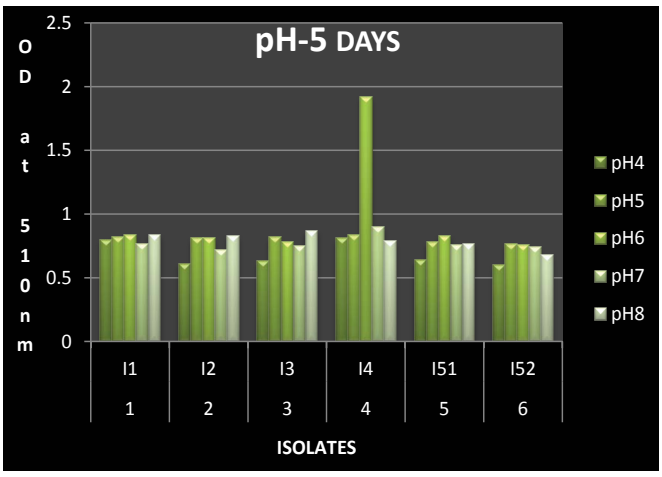

Figure 6: The best pH for enzyme activity was determined by enzyme assay at different pH levels (4, 5, 6, 7, 8). In order to find the effect of pH also 0.5 ml of carboxymethyl cellulose is used as substrate for 5 days.

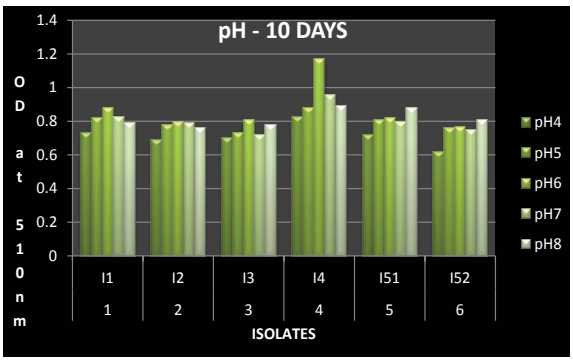

Figure 7: The best pH for enzyme activity was determined by enzyme assay at different pH levels (4, 5, 6, 7, 8). In order to find the effect of pH also 0.5 ml of carboxymethyl cellulose is used as substrate for 10 days.

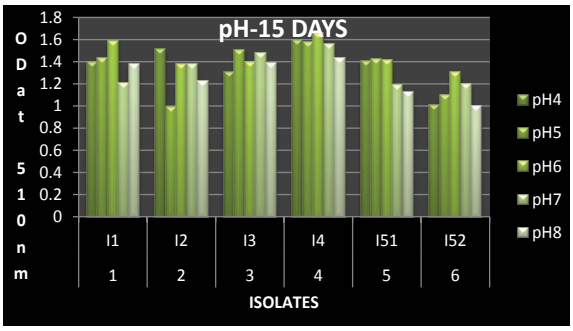

Figure 8: The best pH for enzyme activity was determined by enzyme assay at different pH levels (4, 5, 6, 7, 8). In order to find the effect of pH also 0.5 ml of carboxymethyl cellulose is used as substrate for 15 days.

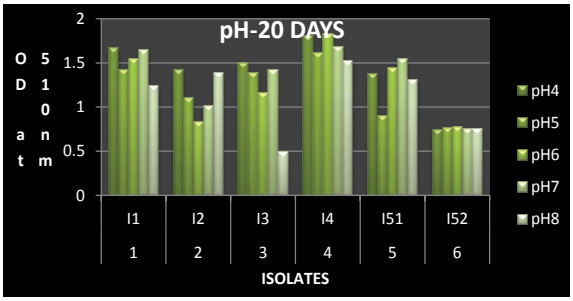

Figure 9: The best pH for enzyme activity was determined by enzyme assay at different pH levels (4, 5, 6, 7, 8). In order to find the effect of pH also 0.5 ml of carboxymethyl cellulose is used as substrate for 20 days.

Conclusion

The bacterial species were isolated from higher termite soil. The six isolates after pure culturing, biochemical characterizations were carried out. The efficiency of bacterial species to produce CMCase was analyzed by screening. In screening the I4 isolate produced more zone of clearance so the organism (I4) utilizes the substrate 1.6 cm, when compared to other isolates. After the screening the optimizations of conditions (pH and temperature) were recorded for 20 days with 5 days of intervals. The I4 isolates at fifteenth day at pH 6 and temperature 40°C were observed to be more efficient and the I4 isolate was identified as *Bacillus* sp. Thus *Bacillus* sp has more potential to produce CMCase enzyme when compared to other isolates. Since CMCase has great applications in food and pharmaceutical industries. These findings suggest that *Bacillus* sp at specific pH and temperature were found to be more potential.

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