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Exploring and Optimizing the Termite Hill Bacterial Diversity for Cellulase Production

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ABSTRACT

Cellulose is the integral component of lignocellulosic biomass which is found abundantly in nature. The lignocellulosic bioconversion is an emerging technology which utilizes microbes and their enzymes for serving the scientific community. In this study, about 126 isolates were isolated from the termite mound soil among which 81 isolates produced cellulase enzyme. Three isolates B12, B25 and B64 exhibited maximum enzyme activity and chosen for optimization parameters such as temperature, pH and incubation time. The isolates B12, B25 and B64 were identified based on 16S rRNA sequencing as *Mesobacillus jeotgali*, *Lysin, Bacillus fusiformis* and *Bacillus* sp respectively. The temperature, pH and incubation time for maximum cellulase production was optimized from the range and depicted that maximum activity at 40 °C, 7 and 60 hours respectively.

Key words: *Cellulase, Termite mound, Mesobacillus jeotgali, Lysinibacillus fusiformis, Bacillus sp.*

Introduction

Lignocellulose is the most abundant renewable resource which has complex and recalcitrant structure and is turning into the essential resource for the bioconversion process (Li *et al*., 2009). The composition of Lignocellulose varies according to the substrate but the overall fraction consists of Cellulose (40 – 50%), Hemicellulose (25 - 30%) and Lignin (15 – 25%). Boosting the process of lignocellulose degradation is the cutting edge research in the field of environment conservation process.

Enzymes are the bio-catalysts composed of fine protein molecules. The cellulosic biomass can be hydrolyzed by a complex set of enzymes comprised as cellulases. Cellulase enzyme has the ability to depolymerize the complex structure of cellulose into simple fermentable form of sugar (Li *et al*., 2009). It consists of three enzymes exoglucanases, endoglucanases and β glucosidase. Limiting fossil fuels and rapid depletion has led to the concern of environment scientists in unleashing the hidden potential of the microbes. Ultimately the enzymes responsible for the degradation of complex polymers was produced by microbial sources. Cellulases are produced by bacteria and fungi for their carbon metabolism (Immanuel *et al*., 2006). But bacteria is mostly preferred to fungi because of its high growth rate and eventually producing more cellulase enzyme (Sethi *et al*., 2013). Even though there are many techniques developed for treatment of cellulosic degradation, still there are enormous resources yet to be converted. In order to match the global cellulose biomass, enzymes are required in abundant quantities. Microbial enzymes are highly stable and actively produced over a long period of time with respect to the capacity of the microbe to produce specific enzymes (Cherry *et al*., 2003). Microbes can

be easily engineered to increase the quantity of enzyme production which is more useful for industrial scale (Ariffin *et al*., 2006).

The aim of this study is to isolate bacterial strains from termite mound soil which has enzymes associated with termite gut microflora. Eventually screening the isolates for cellulase enzyme activity and the best isolates were optimized for maximum cellulase production.

Materials and Methods

Sample collection

The termite soil was collected from the mound of an active termitorium using a screw auger and stored in a pre-sterilized polythene bag. The samples are then transported to the laboratory and stored aseptically at 4 °C for further analysis. An aggregate of about 57 soil samples were collected from three districts of Tamil Nadu, say Coimbatore, Dindigul and Thanjavur.

Isolation and Primary screening of Cellulase producing Bacteria

The soil samples were serially diluted and by employing pour plate technique on Nutrient Agar media for isolation of cellulase producing bacteria. The plates are eventually incubated for 48 hours at 37 °C. The well-established bacterial colonies were sub cultured on fresh CMC agar plate. The plates are then observed for the clear zone around the bacterial colony. For better visualization of the zone, the plates are flooded with 0.1% aqueous Congo red solution for 15-20 minutes and destained using 1M NaCl solution (Bradner *et al*., 1999). More positively zone producing bacteria are selected for further analysis.

Cellulase Enzyme assay

The selected isolates were inoculated in Carboxy methyl cellulose broth and incubated for 72 hours at 37 °C in a shaker. Cell free supernatants are obtained by centrifuging the culture at 6000 xg for 15 minutes. The crude enzyme obtained was used for estimating the amount of reducing sugars released as proposed (Miller, 1959). One cellulase enzyme unit is defined as the amount of enzyme, which released 1 µmol of glucose per ml per minute.

Molecular Identification of Bacterial Isolate

The bacterial isolates of the pure culture were grown overnight in Lysogenic broth (LB) for the bacterial DNA isolation. The bacterial genomic DNA was isolated using CTAB method. DNA extracted from the bacteria was subsequently amplified using a PCR thermocycler. 20µl reaction mixture containing the universal primer 27F (5′ AGAGTTTCCTGGCTCAG 3′) and 1492R (5′ ACGGCTACCTTGTTACGATT 3′) (Lane, 1991) for amplification. The PCR product was verified with agarose gel electrophoresis containing 2.5% agarose and was analyzed with DNA marker of 100bp size. The amplified PCR product was then sequenced by Sanger sequencing in order to get the raw sequence information. Processed sequence was compared against NCBI BLAST to obtain the similar sequences for identification of the bacterial species.

Optimization of parameters for Enzyme production

The parameters handled for optimization aiming at maximum enzyme production were pH, temperature and the incubation time. The most fitting pH was regulated by adjusting the pH of the media which ranges from 3.0 to 9.0. Productive temperature for maximum enzyme production was carried out at altered temperature range of 20 – 50 °C. The process of cellulase production was continued till 96 hours and the cellulase activity was determined at every 12 hours.

Results and Discussion

Isolation and Primary screening of Cellulase producing Bacteria

Bacteria are the good agents in decomposing organic matter particularly cellulosic biomass (Lynd *et al*., 2002). The selective media containing cellulose as the sole carbon source was utilized for the isolation of cellulase producing bacteria. A total of 126 bacterial isolates were obtained from 57 samples and purified isolates were maintained at -80 °C to carry out further studies. The primary screening studies showed the clearance zone around the bacterial colonies which was taken into account. Colonies producing clear zone of ≥7.5 mm diameter were chosen for enzyme production studies. About 45 isolates were eliminated in primary screening. Isolate B12 produced the maximum clear zone diameter of 21mm followed by B64 (19mm) and B25 (18mm).

Cellulase Enzyme assay

Cellulase activity was quantitatively estimated for

the primary screened isolates using carboxy methyl cellulose as the source of carbon and the enzyme activity was calculated. Isolate B12 produced the maximum enzyme activity of 0.771 ± 0.01 IU/ml/min followed by B64 (0.643 ± 0.03 IU/ml/min) and B25 (0.627 ± 0.01 IU/ml/min). This implies that these isolates produced appreciable quantity of enzyme required for the bioconversion of the cellulosic material. The results of cellulase activities of bacterial isolates have been tabulated in Table 1. Among the tested bacterial isolates obtained from the soil, the isolate B7 (0.440 IU/ml/min) showed maximum enzyme activity, followed by B20 (0. 0.357 IU/ml/min), B37 (0.410 IU/ml/min) and B49 (0.334 IU/ml/min) (Patagundi *et al*., 2014).

Table 1. Cellulase activity of the primary screened bacterial isolates

Isolate Name	Mean ± SE	Isolate Name	Mean ± SE
B1	0.340 ± 0.03	B57	0.260 ± 0.04
B2	0.192 ± 0.00	B59	0.243 ± 0.02
B3	0.164 ± 0.02	B60	0.153 ± 0.01
B4	0.247 ± 0.01	B61	0.327 ± 0.01
B6	0.183 ± 0.02	B64	0.643 ± 0.03
B7	0.297 ± 0.01	B66	0.277 ± 0.01
B9	0.181 ± 0.01	B67	0.333 ± 0.02
B10	0.281 ± 0.01	B68	0.216 ± 0.02
B11	0.356 ± 0.02	B70	0.189 ± 0.03
B12	0.771 ± 0.01	B71	0.353 ± 0.04
B13	0.325 ± 0.02	B72	0.268 ± 0.01
B14	0.199 ± 0.01	B73	0.248 ± 0.01
B15	0.178 ± 0.02	B75	0.155 ± 0.02
B17	0.256 ± 0.01	B76	0.172 ± 0.02
B18	0.291 ± 0.01	B78	0.215 ± 0.01
B19	0.249 ± 0.02	B79	0.195 ± 0.01
B21	0.278 ± 0.02	B81	0.340 ± 0.02
B22	0.387 ± 0.01	B82	0.224 ± 0.02
B23	0.268 ± 0.02	B85	0.239 ± 0.02
B25	0.627 ± 0.01	B87	0.256 ± 0.02
B26	0.153 ± 0.02	B88	0.262 ± 0.02
B28	0.248 ± 0.02	B90	0.167 ± 0.03
B29	0.258 ± 0.01	B93	0.269 ± 0.00
B30	0.333 ± 0.02	B94	0.172 ± 0.02
B32	0.340 ± 0.03	B95	0.191 ± 0.02
B33	0.139 ± 0.02	B98	0.229 ± 0.01
B34	0.149 ± 0.01	B100	0.274 ± 0.02
B35	0.175 ± 0.01	B101	0.254 ± 0.03
B37	0.228 ± 0.02	B104	0.347 ± 0.02
B38	0.329 ± 0.02	B106	0.164 ± 0.02
B41	0.267 ± 0.02	B109	0.184 ± 0.02
B43	0.257 ± 0.01	B111	0.208 ± 0.03
B44	0.355 ± 0.04	B112	0.269 ± 0.01
B46	0.234 ± 0.03	B114	0.271 ± 0.02
B48	0.177 ± 0.02	B117	0.323 ± 0.02
B49	0.330 ± 0.01	B118	0.217 ± 0.03
B50	0.211 ± 0.02	B119	0.211 ± 0.03
B52	0.203 ± 0.03	B122	0.237 ± 0.03
B53	0.255 ± 0.02	B123	0.234 ± 0.02
B55	0.291 ± 0.01	B125	0.183 ± 0.01
B56	0.240 ± 0.02		

Molecular identification of bacterial isolates

The three isolates B12, B25 and B64 were chosen for molecular identification since it showed the maximum enzyme activity at standard conditions. Partial sequence analysis of 16S rRNA sequence depicts 100% similar sequences in NCBI database. The isolate B12 was *Mesobacillus jeotgali* (NCBI Accession Number: MT815463), B25 was *Lysinibacillus fusiformis* (NCBI Accession Number: MT815468) and B64 was found to be *Bacillus* sp (NCBI Accession Number: MT815470).

Optimization of parameters for Enzyme production

Optimization was carried out in three isolates *Mesobacillus jeotgali*, *Lysinibacillus fusiformis* and *Bacillus* sp since it had the maximum enzyme production among the bacterial isolates.

Effect of Temperature on Enzyme Production

The most essential parameter in enzyme production is temperature. Temperature differences can bring forth a drastic change in enzyme activity. Hence temperature optimization is very much important to understand the positive extremes of enzyme production. The enzyme activity was found to be the highest at 40 °C for all the isolates and thereafter for every unit increase in temperature up to 45 °C, enzyme activity falls drastically. After 45 °C, the enzyme activity remains still for each unit rise in temperature. The maximum enzyme activities recorded for the isolates *Mesobacillus jeotgali*, *Lysinibacillus fusiformis* and *Bacillus* sp are 0.796 ± 0.01 IU/ml/min, 0.676 ± 0.02 IU/ml/min, 0.715 ± 0.01 IU/ml/min respectively. The enzyme activities are noted and plotted against a graph shown in Figure 1. *Candida tropicalis* YES3 showed maximum cellulase activity at 30 °C (34.203 µmol of glucose min^{-1}) (Geetha Valliammai *et al*., 2021).

Effect of pH on cellulase production

pH of an enzymatic process is wholly dependent on temperature. Hence the temperature of maximum enzyme production (40 °C) was maintained constant

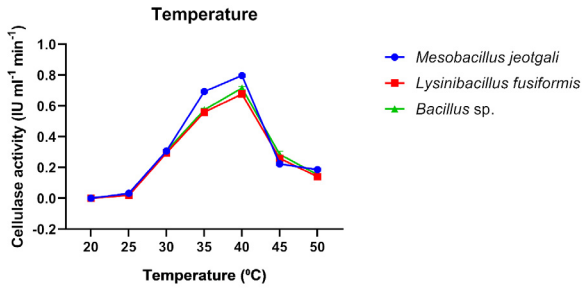

Fig. 1. Effect of Temperature on cellulase production

and pH was altered. It was evident that enzyme activity increased gradually with increase in pH and reached highest enzyme activity at pH 7 and steeps down after pH 7. This shows that most enzyme reactions takes place in a medium of pH 6-7. The maximum enzyme activities recorded for the isolates *Mesobacillus jeotgali*, *Lysinibacillus fusiformis* and *Bacillus* sp are 0.765 ± 0.02 IU/ml/min, 0.645 ± 0.01 IU/ml/min and 0.668 ± 0.02 IU/ml/min respectively. The Enzyme activities are noted from pH 3 – 9 and plotted against a graph shown in Figure 2. The optimum pH for cellulase activity exhibited by *Candida tropicalis* YES3 was 6.5 (35.53 µmol of glucose min^{-1}) (Geetha Valliammai *et al*., 2021). There was no significant difference in the pH change since most isolates were reported to be efficient in the pH range of 6.5 to 7.5.

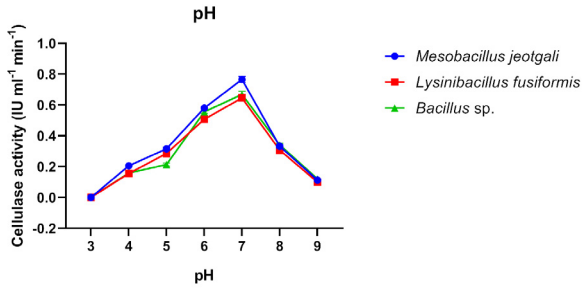

Fig. 2. Effect of pH on cellulase production

Effect of Incubation time on cellulase production

Enzyme production is also governed by incubation time. The optimal time of incubation will result in maximum enzyme production. Enzyme activities were noted for different incubation time, say 0 to 96 hours. This shows that the enzyme activity increases gradually from 0 hours of incubation and reaches its maximum enzyme activity at 60 hours. Thereafter the enzyme activity decreases gradually and almost zero in 96 hours after incubation. The maximum enzyme activities recorded for the isolates *Mesobacillus jeotgali*, *Lysinibacillus fusiformis* and *Bacillus* sp are 0.774 ± 0.01 IU/ml/min, 0.635 ± 0.01 IU/ml/min and 0.611 ± 0.01 IU/ml/min respectively. The decrease in the enzyme activity is due to the depletion of nutrients essential for growth and physiological activity of bacteria. The enzyme activities are noted and plotted against a graph shown in **Figure 3.** Cellulase activity of *Candida tropicalis* YES3 was maximum on day 3 (72h) (33.828 µmol of glucose min^{-1}) (Geetha Valliammai *et al*., 2021). On comparing the potential isolates with characterized yeast isolates, longevity of cellulase production to reach the maximum efficacy was noted in bacterial isolates. But, the biomass obtained will be the added advantage when compared to the yeast isolates and the upscaling would be comaparatively convenient for enzyme production.

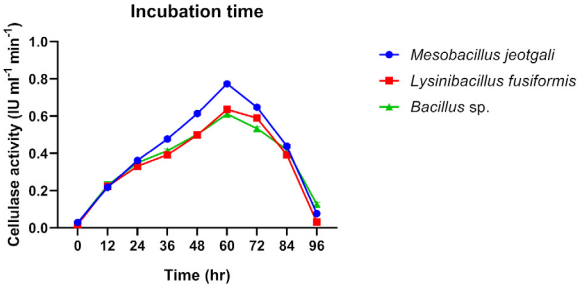

Fig. 3. Effect of Incubation time on cellulase production

Conclusion

There are numerous microorganisms found to degrade and metabolize the cellulosic material as the sole carbon source. Increasing urbanization and industrialization has positive impact towards the environment with abundant cellulosic biomass without any practical applications since it takes centuries to be converted into biofuel. With the aid of the cellulase producing bacteria and the technology of genetic engineering, the ability of a microbe to produce cellulase can be elevated beyond maximum. Thereafter comes the process of bioconversion and production of biofuels from the cellulosic waste biomass. The bacterial isolate *Mesobacillus jeotgali* expressed its maximum potential of producing cellulase enzyme among the bacterial isolates from Termite mound soil. Further improvisation can brought to this study by analyzing the extent of bioconver-

sion and estimation of the biofuel production on which the future world relies upon.

Conflicts of Interest

The authors declare no conflicts of interest/ competing interests.

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