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Qualitative and quantitative assessment of cellulolytic enzyme production in the microbial isolates from environmental samples

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Abstract
The biological treatment of lignocellulosic biomass through enzymatic hydrolysis serves as an ecofriendly and cost efficient method. As these biomass is the most abundant renewable resource which lacks proper utilization and disposal. This study was conducted to isolate and screen the presence of cellulosic enzymes from the microbes of natural ecosystem under laboratory condition. The microbes were isolated from elephant dung, forest soil, termites (gut) collected from Anaikatty forest range of Coimbatore district. Microbes were screened qualitatively and quantitatively for the presence of endoglucanase, exoglucanase, β-glucosidase enzymes. Among the total microbes recovered, one bacteria (BT4), one action bacteria (AS3)and two fungal isolates(FE5,FE6)showed maximum cellulolytic efficiency during qualitative screening, those were further subjected for quantitative estimation of endoglucanase, exoglucanase, β-glucosidase activity.

Keywords: Biomass, microbes, cellulase, hydrolysis

1. Introduction
With the increase in human population and food consumption the lignocellulosic biomass like residues of rice, wheat, maize straw, etc. are generated enormously. Improper way of disposal of these biomass leads to environmental pollution. Among the prevailing degradation process, biological treatment with the use of microbes to convert lignocellulose biomass is ecofriendly and cost efficient when compared to physical and chemical processing technology.
Lignocellulose is the fundamental structural component of all plants comprising cellulose (35-50 %), hemicelluloses (25-30%), lignin (25-30%) together they form a complex matrix (Betts *et al*., 1991) [1]. In the order of degradation of the components of lignocellulosic material, cellulose was the first structural component that needs to be degraded.
Cellulose is the most abundantly available biopolymers on earth. It is the homopolysaccharide consisting of D-glucose linked by ß-1,4-glycosidic bonds forming microfibrils. Microfibrils are joined by intra and intermolecular hydrogen bonds imparting rigidity, structural and chemical stability. In the stalk, stem and woody parts of the plant, the cellulose content was high, their high molecular weight and crystalline structure makes it insoluble material and poor absorber of water, which in turn limits microbial degradation.
The cellulose degrading hydrolytic enzymesare ubiquitous in nature found in different types of organisms including plants, bacteria, insects and fungi. Various subclass of cellulase enzymes includes(1) endoglucanases that randomly cleave intermonomer bonds (2) exoglucanases to remove mono and dimers from the end of glucose chain (3) β-glucosidase to hydrolase glucose dimers (Malherbe *et al*.,2002) [2]. All the three enzymes are together needed for the complete cellulose hydrolysis resulting in simple sugars formation. Cellulose degrading enzymes have wider applications in industries such as food processing, chemicals, paper and pulp production, detergent, textile, wine etc.,
Naturally, wide variety of microbes have the ability to degrade lignocellulosic biomass with the help of diverse enzymes and mechanisms. In which soft rot fungi and white rot fungi are common degrader, apart from them bacteria belonging to genera *Cellulomonas*, *Pseudomonas*, *Bacillus*, *Streptomyces* etc., were widely reported as potential cellulase producers With mutual interaction the microbes obtain carbon and energy sources through degradation of biomass, they play a key role of carbon recycling in the environment.

Materials and Methods

Collection of environmental samples

The samples such as elephant dung (E), termites (gut) (T) and forest soil(S) were collected from Chinna Thadagam 11°4' N latitude and 76° 52' E longitude, Anaikatti forest range of Coimbatore district and the samples were stored at 4 °C for further study.

Enrichment Isolation of cellulolytic organisms

Cellulolytic degraders in the samples such as elephant dung, forest soil and termites (gut) were enriched and isolated. In the termite sample, gut portion alone was dissected, surface sterilized with ethanol, macerated with 0.9% NaCl and centrifuged from which 5 ml supernatant was taken as inoculum. In the other samples, 5 g was inoculated into the Basal Salt Medium ($NaNO_3$ 2.5 g; KH_2PO_4 2 g; $MgSO_4$-0.2 g; NaCl-0.2 g; $CaCl_2 \cdot 6H_2O$-0.1 g in a liter) containing carboxymethyl cellulose 1% w/v (CMC) as the sole carbon source and incubated for 7 days in shaker cum incubator at 37˚C at 100 rpm, facilitating the enrichment of the cellulolytic degraders. After the incubation period, employing serial dilution the enriched inoculum was plated in CMC agar medium. Based on the morphological characteristics, distinct bacteria (B), action bacteria (A) and fungal (F) colonies were picked and sub cultured using the CMC agar medium and further axenic culture was obtained by repeated sub culturing in the same medium and the isolates were stored in glycerol stocks at -80^o C for further studies.

Qualitative screening for cellulolytic property

The confirmation test for the presence of cellulase enzymes from the isolates was done by preliminary screening methods. The isolated colonies were grown individually in CMC liquid medium at 28±2˚C for 2, 3, 5 days for bacteria, fungi and action bacteria respectively. About10µL inoculum was spot drie dat the center of CMC agar medium and incubated for 2, 3, 5 days at 28±2˚Cfor the growth of bacteria, fungi, action bacteria respectively. Then the plates were flooded with 1% Congo red dye and left for 15min and washed with 1M NaCl for des taining the Congo red dye (Gupta *et al*.,2011) [3]. The efficiency of hydrolysis was calculated using the following formula,

$$\text{Cellulolytic efficiency} = \frac{\text{Clear zone diameter} - \text{Colony diameter}}{\text{Colony diameter}} \times 100$$

Quantitative estimation of cellulolytic enzymes

Mass multiplication, crude enzyme extraction and enzyme assays

The isolates were inoculated into CMC liquid medium for cellulase assay and incubated for 2, 3 and 5 days for bacteria and fungi. The inoculum was centrifuged at 10,000 rpm for 10 min at 4˚C. The pellets were discarded and the supernatant portion assumed to contain the extracellular cellulolytic crude enzymes that were used for estimation of enzyme production.

Endoglucanase assay

Endoglucanase activity was estimated employing Mandels *et al.* (1976) [4] method with the substrate modified to CMC instead of filter paper. The cellulolytic activity was measured using UV Spectrophotometer, where in the reaction mixture contained 1.8 ml of extracted crude enzyme, 1% w/v CMC dissolved in 0.1M citrate buffer maintained at pH 4.8, from which 1.8ml solution was added to crude enzyme sample containing test tubes and incubated for 30min at 50˚C. The reaction was arrested by the addition of 3mldi-nitro salicylic acid and placed in water bath at 100˚C for 5min. Blank was simultaneously prepared by adding all reagents except crude enzyme. When the test tubes was still warm, 1ml of Rochelle salt solution was added in all the tubes. The reducing sugar released as the produce of the enzyme activity was measured at the optical density of 540nm. The enzyme activity was expressed as one micromole of glucose released per minute and compared with glucose standard graph(Ghose 1987) [5].

Exoglucanase assay

Cellobiohydrolase's exoglucanase activity was measured adopting Mandels *et al.* (1976) [4], where in the reaction mixture contained 0.2 ml crude enzyme collected from the supernatant, 1.5 ml of substrate obtained by dissolving 0.4% cellobiose in 0.1M citrate buffer of pH 4.8 and incubated at 50˚C for 30min. The reducing sugar was measured at the optical density of 540nm using Spectrophotometer. The result was expressed as one micromole of glucose released per minute

β- Glucosidase assay

The β- glucosidase activity was measured using p-nitrophenyl-β- D-glucopyranoside (pNPG) as the substrate (Berghem *et al*.,1974) [6]. The cultures in the liquid CMC medium was centrifuged at 13,000 rpm for 4 min and the supernatant was incubated in 50 mM citrate buffer with 5 mMpNPG as substrate at pH 5.0 and a temperature of 50˚C for 30 min. The reaction was arrested by adding 10 % sodium carbonate which was followed by the release of p- nitrophenol from pNPG, that was in turn detected at 405nm optical density using UV-Spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme required to release 1 micromole of p-nitrophenol per minute.

Results and Discussion

Isolation of cellulolytic microbes

Enrichment favoured the growth of natural microorganisms. Plating the samples of basal enrichment medium into CMC agar plates, a total of 24 microbial strains were recovered. The colonies were selected based on distinct morphological characters. Among them 6 strains of fungi (FE1, FE2, FE3, FE4, FE5, FE6) and 4 strains of bacteria (BE1, BE2, BE3, BE4) were isolated from elephant dung. 3 strains of fungi (FT1, FT2, FT3) and 3 strains of bacteria (BT1, BT2, BT3) isolated from termites (gut) and 2 strains of fungi (FS1,FS2), 2 strains of bacteria (BS1, BS2) and 4 strains of action bacteria (AS1,AS2, AS3, AS4) were isolated from forest soil.

In recent years, action bacteria was proved to be potential cellulose degraders which was supported by the study of Větrovský *et al.* (2014) [7] by isolating seventy six actinobacterial strains from soil in which 31% of actinobacteria produced both cellobiohydrolase and 1,4-β-glucosidase enzyme. A study by Tsegaye *et al.* (2019) [8] revealed that microbiome from termite (gut) found to possess potential cellulose degrading population. Similarly, eight new fungi isolated from Asiatic elephant dungsamples proved to be the potential cellulolytic degraders (Farouq *et al.*, 2012) [9].

Screening of isolates for cellulolytic activity

The Congo red plate assay was done for the 24 isolates, of which 10 isolates showed positive results by formation of halo zone around the colony using Congo red dye indicator.

Based on the colony diameter and halo zone formation, the cellulolytic efficiency was calculated and provided in the Table.1.Among the isolates, BT4 bacterial culture isolated from termite has the maximum cellulolytic efficiency of 65% and a total of 4 isolates (BT4,FE1, FE6, AS3) showed high cellulolytic efficiency was taken for further enzyme estimation. This result correlated well with the study of Mahalingam *et al*. (2014) [10], in that study, solubilization index was measured ascellulolytic efficiency among fungal strains, in particular *Aspergillus fumigates* had the highest solubilization index of 57.96 ± 0.02.

The halo zone formation indicates the positive result for the cellulolytic activity, in contrast the congo red dye still remaining in the plate reveals the presence of non hydrolysed β-1,4-D-glucosidic bonds (Lamb *et al.*, 2005) [11]. In a previous study, preliminary screening was opted as a vital method for selecting potential 14 cellulolytic bacteria from 30 isolates (Zhang *et al.*, 2017) [12].

Table 1: Cellulolytic efficiency of microbial isolates

Microbial Isolates	Cellulolytic efficiency (%)
Bacteria	
BE2	15
BT3	26.9
BT4	65
Fungi	
FE1	55
FE5	10
FE6	60
FT2	12
Actino bacteria	
AS2	15
AS3	50

(B-Bacteria, F-Fungi, T-Termite, E-Elephant dung, A-Actino bacteria, S-Soil)

Enzymatic profile of isolates

Primary screening provides information on the overall cellulolytic activity of the organism, whereas secondary screening was a quantitative estimation of the presence of cellulase enzymes in isolates.

The previously screened four isolates were subjected for quantitative enzyme activity studies. After mass multiplying the cultures in the CMC liquid medium for 8 days, at regular intervals, the supernatant collected by centrifugation contained the crude enzyme portion that was subjected for quantitative estimation studies at regular intervals.Among them, isolateFE6 showed the maximum endoglucanase activity of 6.032 IU/ml on 5th day of incubation (Fig.2).The exoglucanase activity recorded highest (4.142 IU/ml) for the isolate FE5 on 6th day of incubation (Fig.3) and the β-glucosidase activity recorded maximum (5.024IU/ml) for the isolate BT4 on 4thday of incubation(Fig.4).Overall, all the selected isolates showed an increase in cellulase enzyme activity over a period of 4 to 6 days followed by a gradual decline.

The active period (up to 7 days) for cellulase production corroborates with a previous study in which CMC and filter paper were used as substrate, in this study also the enzyme secretion was noted upto 8 days of incubation period (Adsul *et al*., 2004) [13]. A study by Deschamps *et al*., (1985) [14] observed that using filter paper as cellulose substrate, *Trichodermaharzianum* produced maximum cellulolytic activity of 11 IU /g after 3 days of incubation. Similarly, highest production of cellulolytic enzymes was noticed in *Trichodermaressei* over an incubation period of 5 days with Endoglucanase, Exoglucanase and β- glucosidaseactivity 4.692±0.04, 2.759±0.04, 6.01±0.06respectively(Bilal *et al*., 2015) [15].

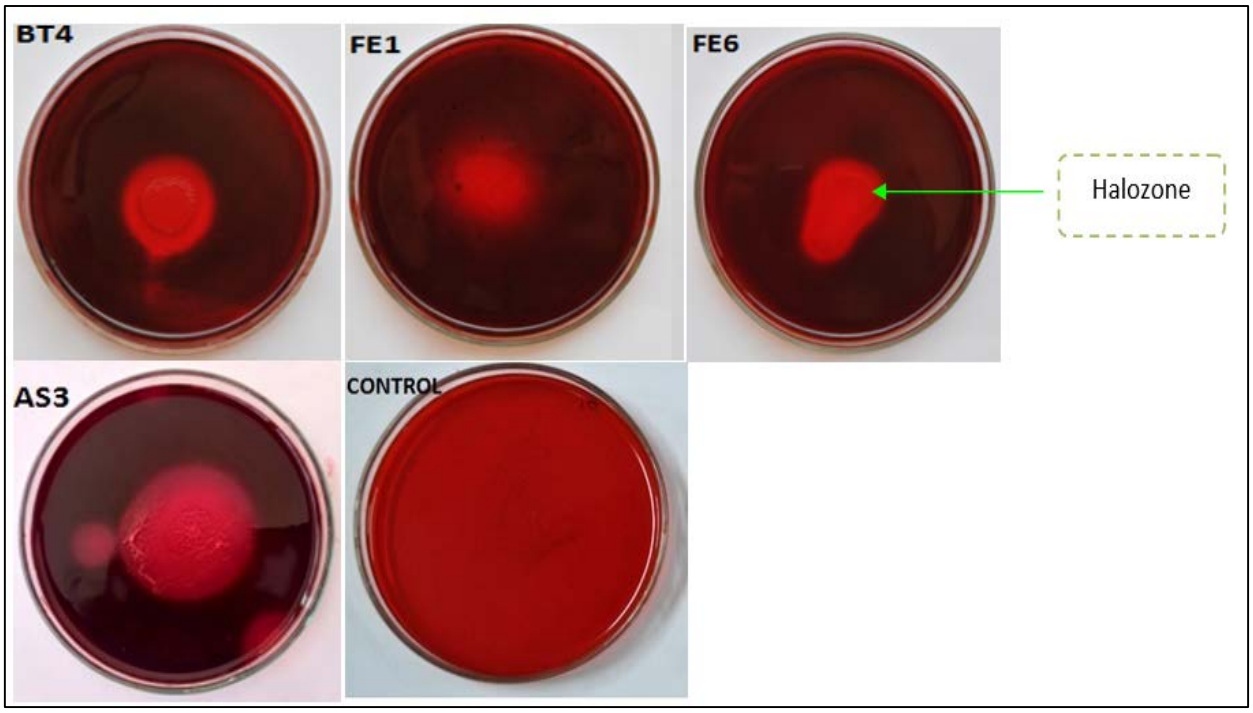

Fig 1: Screening of cellulose degrading bacteria, fungi, actino bacteria

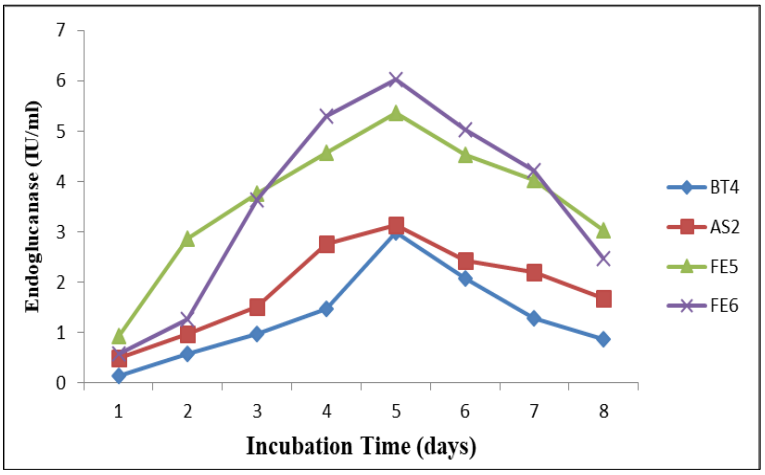

Fig 2: Endoglucanase enzyme activity of isolates over different time period

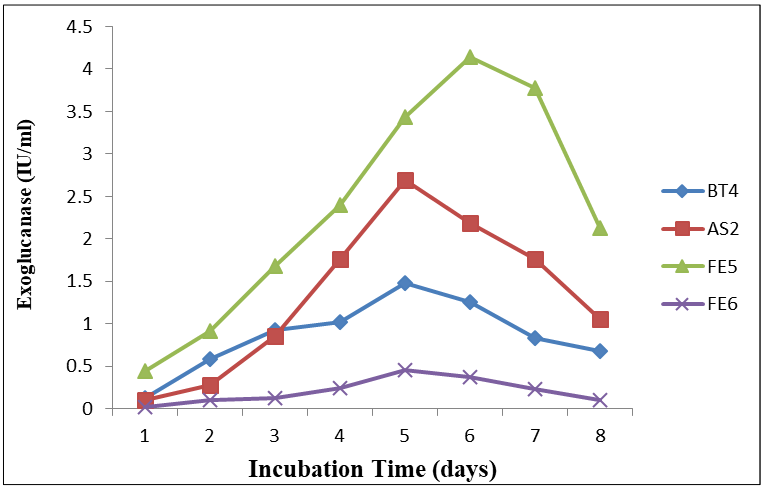

Fig 3: Exoglucanase enzyme activity of isolates over different time period

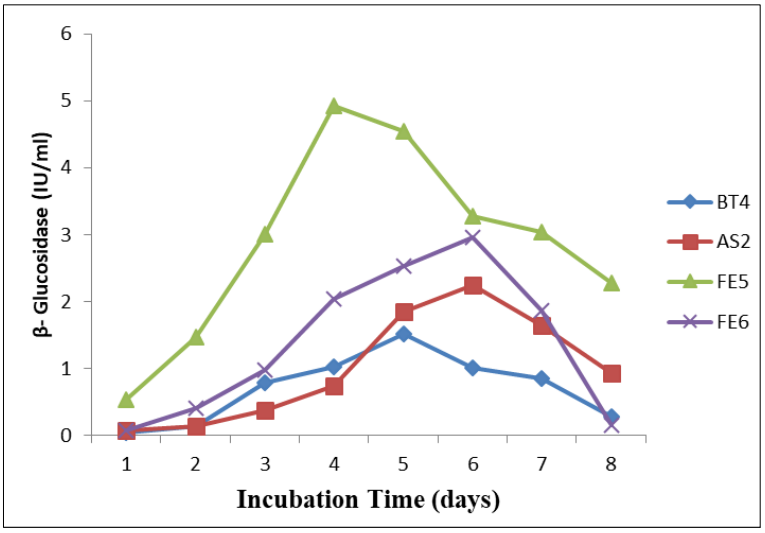

Fig 4: β- Glucosidaseenzyme activity of isolates over different time period

Conclusion

This study highlights that organisms isolated from various forest resources including elephant dung, degraded wood, termites (gut) found to harbour cellulolytic microbial population. Four isolates comprising of 1bacteriumisolated from termite gut, 1 actino bacterium isolate recovered from forest soil, 2 fungi isolated from elephant dung origin were proved to be cellulolytic in nature through qualitative and quantitative measurements. In particular, quantitative measurements proved that cellulolytic activity was mediated through endoglucanase, exoglucanase and beta glucosidase activity. Screening of microbial population from natural sources remains as the vital step for further research in production of bio based products through biomass conversion.

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