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Bioprospecting of microorganisms with lignocellulolytic enzyme activity for leaf litter degradation

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Abstract
This study aims to isolate cellulose and lignin degrading microorganisms which are capable of degrading lignocellulosic biomass in an ecofriendly nature. A total of 160 cultures were isolated and screened for their cellulolytic, ligninolytic activity. Those cultures were forwarded for lignocellulolytic enzyme assays. Among the bacterial isolate, TER3 showed the maximum number of enzyme activity in cellulase, PPO and laccase (6.024 U/ml, 0.963 U/ml and 0.215 U/ml respectively). E-PW1 isolates were observed, to the production of PPO (0.237 U/ml), MnP (0.007 U/ml) and LiP (0.242 U/ml). E-MUS1 isolate also showed good activity in cellulase (2.875 U/ml), LiP (0.240 U/ml) production. In the case of fungal isolates TER1 exhibited the highest activity in PPO (2.468 U/ml), laccase (0.053 U/ml) and LiP (0.244 U/ml) production, COM3 isolate recorded activity of 0.490 U/ml in PPO and 0.240 U/ml in LiP followed by E-PST3 (0.013U/ml in laccase, 0.203U/ml in LiP). In Actinobacterial culture CP4 showed higher activity in cellulase production (5.140 U/ml), laccase (0.124 U/ml) production, E-PST4 showed 0.008 U/ml in laccase, 0.149U/ml in LiP production and COM2 in cellulase (2.66U/ml). Selection of cultures were undertaken based on the number of enzymes produced. The selected 9 cultures were considered as the best lignocellulose degrading microorganism that could produce all the desired enzymes for effective degradation of lignocellulosic biomass.

Keywords: Lignocellulosic biomass, cellulase, polyphenol oxidase, lignin peroxidase, manganese peroxidase, laccase

1. Introduction
The generation of large amounts of agricultural wastes has become a serious problem in recent years and a source of environmental concern. Lignin consists of numerous biologically stable linkages and having the most complex nature structurally. It is the most recalcitrant compound, possesses a high molecular weight (Perez *et al*., 2001) [11]. Due to its complex organization, breaking of lignocellulosic material is extremely difficult (Himmel *et al*., 2007) [6]. Some of the microorganisms are identified as lignocellulosic degrading organisms. Fungi and bacteria are the major two types of microorganisms which play a predominant role in the biodegradation of lignin. Actinomycetes and Proteobacteria are the two primary classes of lignin-degrading bacteria. Lignin is degraded by microbial enzymes into non-toxic compounds (Huang *et al*., 2013) [7].
Cellulase, hemicellulases, xylanases and lignin modifying enzymes that form a combination, which able to react on lignocellulosic materials, for improving its hydrolysis. Lignin peroxidase (LiP) was reported to be the first enzyme that degrades lignin. It was initially discovered in 1983 (Shi *et al*., 2013) [5]. Although non-biological treatment of lignocellulosic wastes was an effective step, it was not an environmentally favourable technique (Kong *et al*., 2017) [8]. Thermochemical treatments can liberate toxic and corrosive like derivates (black liquor) during the degradation of lignin (Fang *et al*., 2018) [4]. Numerous harmful and scathing side-effect compounds (dark alcohol) can be made by thermochemical medicines because of lignin corruption. Therefore, the main aim of this investigation was to isolate novel microbial strains of bacteria, fungi and actinobacteria from various sources. Screening their lignocellulolytic ability and also determining the enzyme activity for assessing their biodegradation capability, which can be the cost effective method for bioconversion of lignocellulosic biomass.

2. Materials and Methods

2.1 Collection of Sources

Twenty different lignocellulosic sources like degraded wood (DW), termite (TER), cow dung (CD), paddy soil (PS), paddy straw (PST), termite soil (TS), mushroom (MUS), coir pith (CP), paper waste (PW), compost (COM) were collected from Agricultural College & Research Institute, Madurai as source material for isolation. Out of twenty, ten sources were enriched in BSM (Basal Salt Media).

2.2 Isolation of Microorganisms

Bacterial, fungal and actinobacterial cultures were isolated from both raw and enriched samples using the serial dilution technique. 1g of sample was taken, serially diluted up to 10^{-6} dilution. The samples are then plated on Nutrient agar media (NA), Rose Bengal agar media (RB), Kenknights media (KK) and incubated for 2, 4, 7 to 10 days for bacteria, fungi and actinobacteria. Purification of the cultures was done to obtain pure cultures and stored in refrigerated conditions.

2.3 Qualitative estimation

2.3.1 Cellulolytic assay

Isolates were plated on the CMC (Czapek mineral salt media) and incubated for 5 days. Followed by plates were flooded with 0.5% of Congo red solution and leave the plates for 15 mins. Then wash using 1M NaCl solution to destain. The cellulolytic activity was indicated in cultures that form clear zone (Teather *et al*., 1982) [17]

2.3.2 Ligninolytic assay

The isolates that tested positive for cellulolytic activity were forwarded to ligninolytic activity testing. Cultures were plated on MSM (Minimal salt media) with 1% Alkali lignin as the sole carbon source and incubate for 5 days. The cultures that were grown on this media are considered as effective lignocellulolytic cultures (Chandra *et al*., 2008).

2.4 Quantification of the produced enzymes

2.4.1 Cellulase

The Cellulase activity was determined using carboxymethyl cellulose as substrate in the reaction mixture with 0.05 ml enzyme extract, followed by incubation for 15 min at 55 °C. After the incubation period, the reaction was terminated by adding 1.5 ml of 3, 5-dinitrosalicylic acid (DNS) reagent. Reducing sugars were estimated with 3, 5-dinitrosalicylic acid (Miller, 1959) [9] using glucose as standards. One unit of enzymatic activity is defined as the amount of enzyme that releases 1 µmol reducing sugars (measured as glucose) per ml per minute.

2.4.2 Polyphenol Oxidase

The reaction mixture consists of 2.5ml of 0.1M phosphate buffer pH 6.5, 0.3ml of catechol solution (0.01M) and 0.2ml of enzyme extract. After 5 min of incubation, absorbance was measured at 495nm. Enzyme activity was expressed as changes in absorbance min^{-1} mg^{-1} of protein (Sadasivam, 2004)

2.4.3 Lignin Peroxidase (LiP)

The enzyme assay was carried out in a standard reaction mixture consisted of 1 ml of 125 mM sodium tartrate buffer (pH 3.0), 500 µl of 2 mM hydrogen peroxide solution, 500µl of 10 mM veratryl alcohol, and 500 µl of the culture filtrate. The absorbance of the solution was monitored at 310 nm. One unit of enzyme activity was measured by one mole of veratraldehyde produced per minute per ml of the culture filtrate (Daljit S. Arora, 2001) [1]

2.4.4. Manganese Peroxidase (MnP)

Reaction mixtures consists of 0.1 mM MnSO4, 0.1 mg/ml of phenol red, 25 mM lactate, 1 mg/ml of bovine serum albumin and 0.5 ml of culture filtrate in 20 mM sodium succinate buffer (pH 4.5) in a total volume of 3 ml. The reaction was started by the addition of hydrogen peroxide to the final concentration of 0.1 mM and was stopped after 1 min by adding 50 µl of 10% NaOH. The absorbance of the solution was measured at 610nm. The activity was expressed as the increase in A610 per minute per milliliter (Glenn and Gold, 1985) [5].

2.4.5 Laccase

The laccase activity was determined by the oxidation of tannic acid by using laccase production medium. Stock solutions of salts were prepared separately and autoclaved. Then add the respective salt solutions to the medium. 1 mL of isolates from the laccase production medium were transferred to test tubes containing 0.3 percent tannic acid and incubated at 35°C **(**Dastidar *et al*., 2018) [3].

2.5 Statistical analysis

The statistical analysis was done with the AGRES software package (Gomez *et al.,* 1984) [18] at a 5 percent probability level using completely randomized design (CRD).

3. Results and Discussion

3.1 Isolation of cellulolytic and Lignolytic cultures

Based on different colony characteristics, total of 160 cultures including bacteria, fungi and actinobacteria were isolated from varied sources *viz*., paddy straw, termite, cow dung, degraded wood, termite soil, paddy soil, mushroom, paper wastes, compost and coir pith (both raw and enriched). All the isolates were subsequently purified and preserved. Out of those 160 isolates, 71 isolates from enriched samples and 89 isolates were obtained from raw samples.

3.2 Primary screening of isolates for cellulolytic and ligninolytic enzyme

3.2.1 Qualitative cellulase enzyme assay

All the 160 cultures were screened for their cellulase production by growing them on agar plates containing carboxymethyl cellulose as their sole carbon source. Cellulolytic cultures able to produce cellulase enzyme and made clear zone around the colony indicates cellulose degrading capabilities. Out of 160 isolates, 89 isolates showed the zone of clearance on the agar plate. Among the 89 isolates, 20 bacteria, 29 fungi and 40 actinobacteria were tested positive for cellulase assay (Fig 1). The results were found to be conformity with the findings of Neethu *et al* (2017) [10]. They have isolated 103 bacteria from the compost sample and were screened for their production of cellulase by using the CMC plate assay. From 103 cultures, fourteen isolates were selected which showed higher clear zone on CMC plate and forwarded for quantitative screening.

3.2.2 Qualitative lignolytic assay

Screening of lignolytic microorganisms were done using MSM agar media with alkali lignin (L) as their sole carbon source. All the 89 cellulolytic isolates obtained were screened

for lignolytic activity. About 40 isolates showed better growth in alkali lignin media indicates positive for lignin degradation (Fig 2). Similar results were found by Dastidar *et al*., (2018) [3] reported the isolation of ligninolytic microbes from the different environmental niches. The isolates showed varied growth rates in MSML with few having more affinity to lignin. The dye reducing activity of the isolates indicates the presence of the oxidative ligninolytic enzymes showing a high growth rate in MSML had greater efficiency of lignin degradation.

3.3 Secondary screening of isolates

Based on the cellulolytic and lignolytic activity, 40 cultures were progressed for quantitative estimation of enzymatic assays.

3.3.1 Enzyme assays

Among the 9 bacterial isolates, TER3 culture exhibited maximum activity in cellulase production (6.024 U/ml) comparing to other bacterial isolates. In fungal culture, PST5 culture showed the highest cellulase production (19.36 U/ml), PW2 (10.86U/ml) followed by TER1 isolate (10.826 U/ml). Comparing 12 actinobacterial isolates, the CP4 isolate result (5.14 U/ml) revealed a higher amount of cellulase production Overall cellulase production was higher in fungi compared to bacteria and actinobacteria. Pinky Prasad *et al*., (2014) [12] isolated 10 cellulolytic actinomycete strains and analysed the cellulolytic potential of the selected strains showed that the strains CD-3 (0.023 IU/ ml CMCase unit and 0.009 IU/ ml FPase unit) and CD-10 (0.020 IU/ ml CMCase unit and 0.010 IU/ ml FPase unit) were the most efficient cellulose degrading actinomycete strains. Estimated quantitative assay of carboxy methyl cellulase (CMCase) activities exhibited by the positive isolates showed that the highest CMCase was produced by NAA2 0.366 IU/ml (Saini *et al.*, 2016) [14]

Higher polyphenol oxidase activity was observed maximum in TER1 (2.47 U/ml) compared with the actinobacterial CP1 isolate (1.34 U/ml) and bacterial isolate TER3 (0.96 U/ml). Fungal isolate TER1 (0.244 U/ml) showed the highest activity of lignin peroxidase and the lowest activity was recorded in E-COM1 (0.57U/ml). Among the 9 bacterial isolates, E-PW1 isolate showed higher amount of lignin peroxidase activity (0.242 U/ml). In actinobacterial culture, E-PST4 showed the maximum activity of 0.149U/ml. Sondhi *et al.*, 2015 revealed that lignin degrading enzymes like manganese peroxidase was released by *Stenotrophomonas maltophilia* RSI6, *Klebsiella pneumoniae* RSI9, *Bacillus cereus* RSDa2, with activities of 0.067, 0.088 and 0.094 units/min/ml respectively. Lignin peroxidase production was 1.122, units/min/ml for *S. maltophilia* RSI6, while the other two isolates also produced Lignin Peroxidase. The maximum laccase production was recorded in *Stenotrophomonas maltophilia* RSI6 (0.217 units/ min/ml), followed by *Bacillus cereus* RSDa2.

Table 1: Quantitative estimation of enzyme activity for fungal isolates

S. No	Fungal Isolates	Cellulase (U/ml)	Polyphenol Oxidase U/ml	Lignin Peroxidase U/ml	Laccase U/ml	Manganese Peroxidase (U/ml)
1	TER 1	10.826	0.494	0.244	0.053	0.003
2	TER 2	2.844	0.021	0.119	0.009	0.004
3	COM 2	2.110	0.050	0.146	0.007	0.002
4	COM 3	2.722	0.098	0.240	0.011	0.008
5	PW 1	0.520	0.038	0.071	0.003	0.004
6	PW 2	10.856	0.083	0.097	0.004	0.008
7	CD 1	1.560	0.037	0.111	0.004	0.006
8	CD 2	9.602	0.001	0.070	0.004	0.003
9	PST 1	1.651	0.009	0.134	0.004	0.001
10	PST 2	0.336	0.016	0.200	0.009	0.002
11	PST 5	19.358	0.039	0.175	0.009	0.002
12	PST 6	10.398	0.033	0.122	0.005	0.005
13	PST 7	5.474	0.018	0.192	0.011	0.001
14	PST 8	1.315	0.017	0.155	0.008	0.007
15	PS 1	4.006	0.009	0.080	0.002	0.001
16	E-COM 1	7.798	0.024	0.057	0.002	0.006
17	E-COM 3	1.040	0.027	0.082	0.005	0.008
18	E-COM 4	3.517	0.029	0.165	0.007	0.008
19	E-PST 3	2.263	0.050	0.203	0.013	0.005

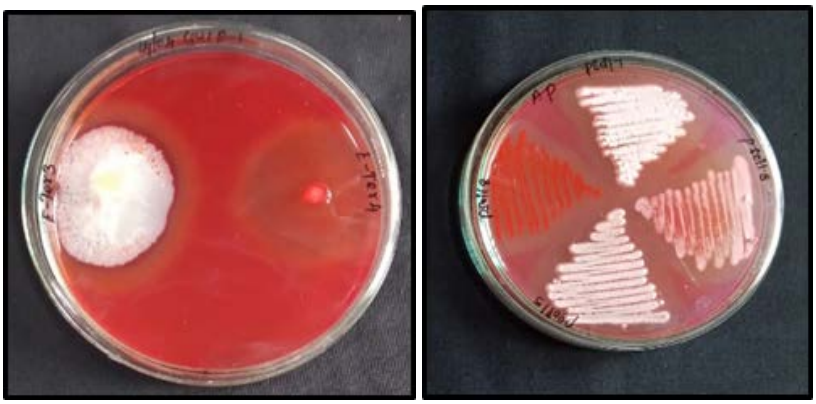

Fig 1: Zone of hydrolysis of different fungal and actinobacterial isolates on CMC agar medium

Fig 2: Bacterial, fungal and actinobacterial growth observed in MSML agar medium

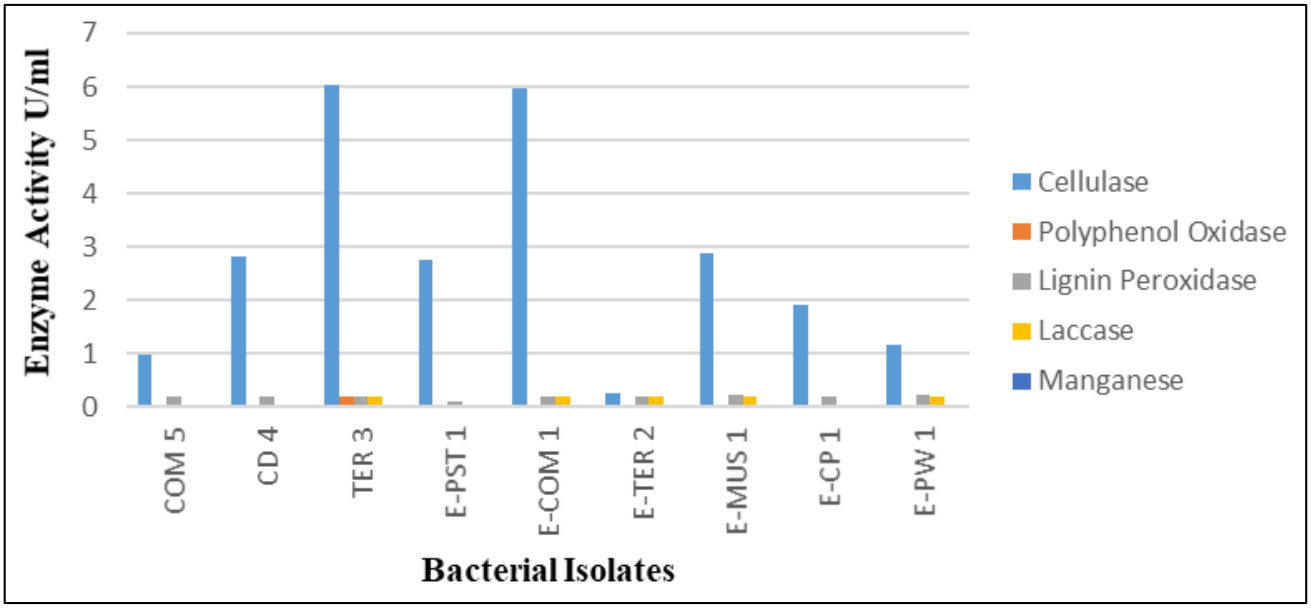

Fig 3: Lignocellulolytic enzyme assay shown by different bacterial isolates

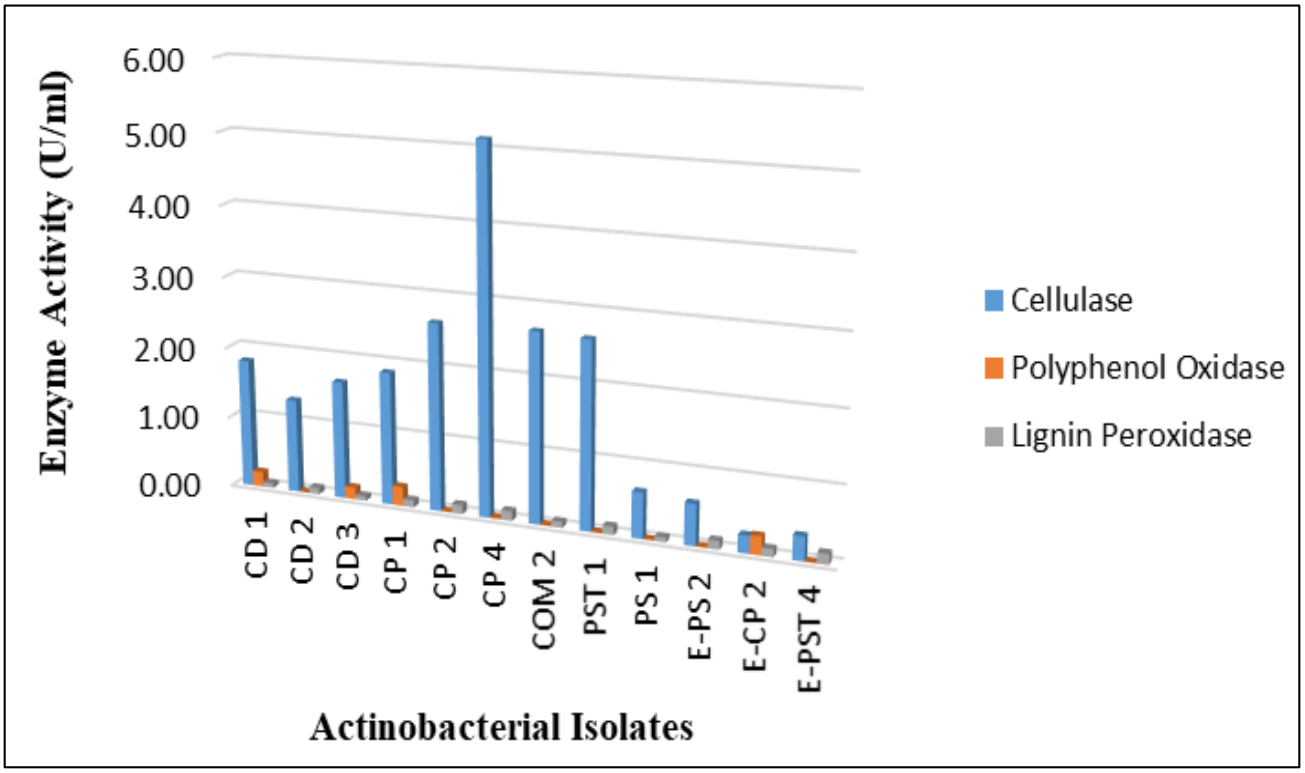

Fig 4: Cellulase, Polyphenol Oxidase and Lignin Peroxidase assays shown by different actinobacterial isolates

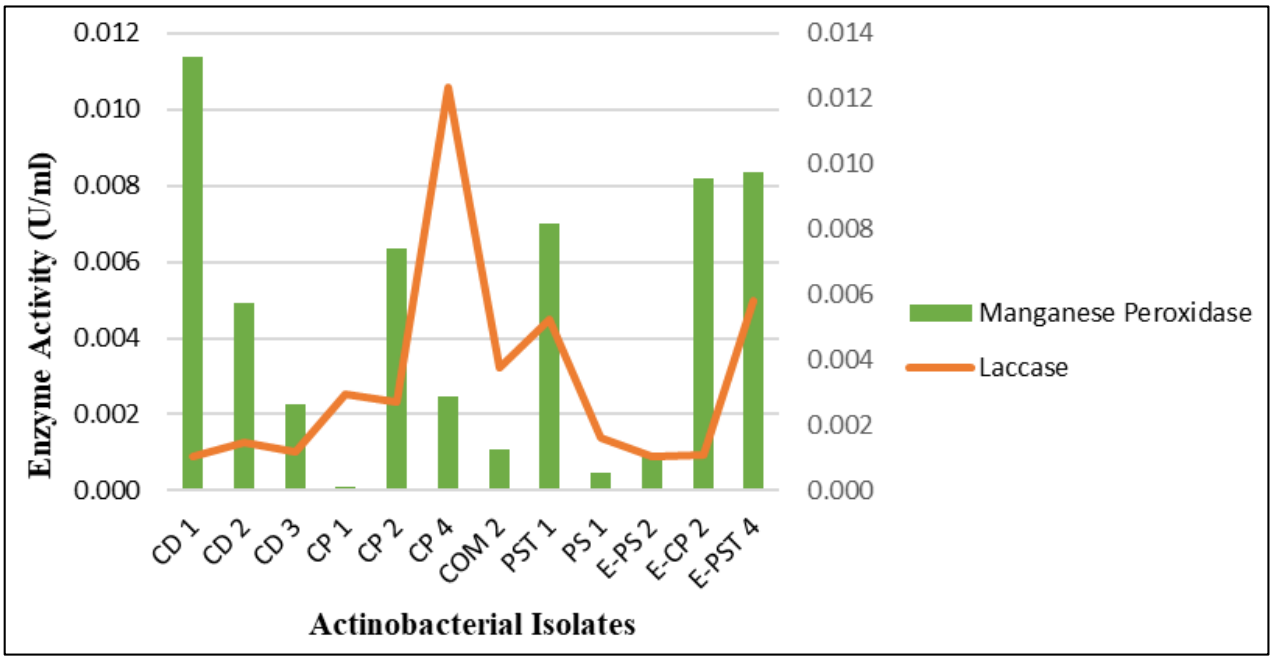

Fig 5: Laccase, MnP assay shown by different actinobacterial isolates

4. Conclusions

Based on the qualitative and quantitative screening of the isolates for lignocellulolytic enzyme activity, it is concluded that none of the single culture could produce all the enzymes. Three cultures, each from bacteria, fungi and actinobacteria were selected as the effective lignin and cellulose degrading microbes. These cultures were pooled together and could be used for preparation of microbial consortium for degradation.

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