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Isolation and Screening of Potential Lignocellulolytic Microorganisms from Different Ecosystems

U. Jagadeesh and R. Muthuraju
Department of Agricultural Microbiology, College of Agriculture, UAS, GKVK, Bengaluru - 560 065
e-Mail : jaggumurali@gmail.com

Authors Contribution

U. Jagadeesh :
Data collection, data analysis, drafting the article & Interpretation;
R. Muthuraju :
Conception of the work, Critical revision of article & final approval.

Corresponding Author :
U. Jagadeesh
Department of Agricultural Microbiology, College of Agriculture, UAS, GKVK, Bengaluru

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Abstract

Lignocellulolytic microorganisms can degrade agricultural biomass with the production of hydrolytic enzymes that degrade recalcitrant lignocellulose. This study aims to isolate lignocellulose degrading microorganisms which are capable of degrading lignocellulosic biomass in an ecofriendly nature. Lignocellulolytic microorganisms were isolated from degraded wood, paddy straw piles, cow dung, forest soil, termite midgut, paddy field soil, tree barks, compost and vermicompost. A total of 173 isolates were obtained and screened according to their qualitative cellulolytic and ligninolytic activities. The superior isolates were then quantitatively assayed for their ability to produce lignocellulolytic enzymes such as FP-ase, CMCase, β-D glucosidase, Xylanase, Manganese peroxidise (Mnp), Lignin Peroxidase (Lip) and Laccase. Isolates were selected based on the number of enzymes produced. The isolates UASFW, UASFK2, UASBW1 and UASAP2 displayed the highest enzyme activities with a CMCase activity of 232.42, 247.19, 218.86 and 83.24 U/mL, respectively. Fungal isolates UASFW (547.23 U/mL) and UASFK2 (621.54 U/mL) exhibited highest Laccase production compared to bacterial and actinobacterial isolates. Overall, cellulase production was higher in fungi as compared to bacteria and actinobacteria. Whereas, Lip and Mnp activity was better in bacteria and actinobateria compared to fungi. The selected isolates (UASFW, UASFK2, UASBW1 and UASAP2) were considered as the best lignocellulose degrading microorganisms that could produce all the desired enzymes for effective degradation of lignocellulosic biomass.

Keywords : Lignocellulosic biomass, Cellulase, Lignin peroxidase, Manganese peroxidase, Laccase

Lignocellulosic waste is considered as a chief component of renewable biomass on the earth, with an annual output of about 1,500 million tons (Xing *et al*., 2020). It is a product associated with agricultural production and straw is one of the primary sources of lignocellulose. However, large amounts of straw are burned or discarded in many countries, while biomass recycling has become an effective method of reducing this straw waste (Zhao *et al*., 2019). This consists of three major components such as cellulose (40–50%), hemicelluloses (25–30%) and lignin (15–20%) (Chaurasia, 2019). Cellulose is the primary polysaccharide as constituent of lignocellulosic material that consist thousands of D-glucopyranose units linked by β-(1–4) glycosidic linkage in unbranched linear chains. Cellulose, hemicellulose and lignin form water-insoluble compact network structure that limits its degradation (Faria *et al.*, 2020). Therefore, pre-treatment (physical, chemical and biological) is required to facilitate fermentable sugar release. The biological pre-treatment (enzymes and cellulolytic microorganisms) remains the best approach to address this issue because it is eco-friendly (Sankaran *et al*., 2020).

Cellulase is a whole enzyme system composed of endoglucanase and exoglucanases including cellobiohydrolases and β-glucosidase (Xue *et al*.,

2020), which break down β-1,4-linkages in cellulose polymer to release glucose units. Many investigators have reported that aerobic and anaerobic bacteria (Mokale *et al*., 2022), fungi and actinomycetes (Houfani *et al*., 2022; Ramesh *et al*., 2020 and Ghosh *et al*., 2021) are good cellulase enzyme producers. These microbes secrete free or cell surface-bound cellulases and exhibit an efficient enzyme decomposition. Cellulases are very successful in the industrial exploitation of the degradation of lignocellulosic biomass (Kakde and Aithal, 2021).

Lignin is an aromatic polymer and one of the primary components of lignocellulose. Lignin is primarily composed of *p*-coumaryl (H), guaiacyl (G) and syringyl (S) monomers and is connected by a β-O-4 bond, β-5 bond and β-β bond (Cajnko *et al*., 2021). Lignin accounts for approximately 20 to 32 per cent of the natural lignocellulose structure and is the second most abundant natural organic polymer, after cellulose. Lignin strengthens the plant structure to prevent cellulose and hemicellulose from being hydrolyzed. The complex of lignin structure also makes it difficult for lignocellulose to be decomposed and transformed by an organism, limiting the efficient use of agricultural waste. Biological treatment is often needed when using lignin-rich agricultural waste, including for papermaking, composting and biogas production (Adarsh and Chandra, 2020).

A few microorganisms can naturally degrade lignin and produce lignin-degrading enzymes, including fungi, actinomycetes and bacteria (Li *et al*., 2020 and Bohacz & Kowalska, 2020). They can secrete extracellular lignin degradation oxidases, which are primarily divided into lignin peroxidase (EC 1.11.1.14), manganese peroxidase (EC 1.11.1.13) and laccase (EC 1.10.3.2) (Adarsh and Chandra, 2020).

The enzymatic degradation of lignocellulosic biomass is important for sustainable utilization of agricultural residues. Hence, production of high efficient and economic cellulolytic enzymes including cellulase, xylanase and glycosyl hydrolases, has received a great attention. The key to using biological methods to decompose lignin is to obtain microbial resources that efficiently decompose and transform lignocellulose and mine the enzymes with lignocellulose degrading ability in microorganisms.

Therefore, the main aim of this investigation was to isolate novel microbial strains of bacteria, fungi and actinobacteria from various sources and 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.

Material and Methods

Collection Sources of Samples

Fifteen different lignocellulosic sources *viz.,* paddy straw, degraded wood, termite gut, cow dung, forest soil, paddy field soil, tree barks, compost and vermicompost were collected from GKVK, Siruguppa, Ujire and Karnataka Compost Development Corporation (KCDC), Bengaluru as source material for isolation of lignocellulosic degrading microorganism.

Isolation of Microorganisms

Bacterial, fungal and actinobacterial isolates were obtained from both raw and enriched samples using the serial dilution technique. One gram of sample was taken, serially diluted up to 10^{-5} dilution. The samples are then plated on Nutrient Agar medium (NA), Potato Dextrose Agar (PDA), Martin's Rose Bengal Agar medium (MRBA), Kenknights medium (KK) and incubated for 2, 4 and 7 to 10 days for bacteria, fungi and actinobacteria, respectively. Purification of the cultures was done to obtain pure cultures and stored in refrigerated conditions.

Qualitative Estimation

Cellulolytic Assay

The isolates were plated on the CMC (Czapek mineral salt medium) and incubated for five days followed by plates were flooded with 0.5 per cent of congo red solution and left for 15 minutes. Then wash using 1M NaCl solution to destain. The cellulolytic activity was indicated in cultures that form clear zone (Teather *et al*., 1982 and Vidyashree & Brahmaprakash, 2018).

Ligninolytic Assay

The isolates that tested positive for cellulolytic activity were forwarded to ligninolytic activity testing. Cultures were plated on MSM (Minimal Salt Medium) with 1 per cent 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).

Quantification of the Produced Enzymes

Determination of Exo β-1,4- glucanase (FP-ase)

FP-ase activity was measured according to Mandels *et al*. (1976) by mixing 1ml of 0.05M citrate buffer (pH 4.8) and 50 mg of Whatmann filter paper no.1 (strips of 1×6 cm) at 50°C for 1 hour. Reducing sugars were measured as glucose according to the method of Miller (1959). One unit of enzyme is defined as the amount of enzyme which released 1 micromole of reducing sugars, expressed as glucose per minute, under given condition.

Determination of Endo b - 1, 4 - Glucanase (CMC-ase)

Carboxymethyl cellulase (CMCase) was measured according to Mandels *et al*. (1976). Reaction mixture (1ml of enzyme solution, 1ml of 1 per cent CMC dissolved in 0.05M sodium citrate buffer pH 4.8) was incubated at 50°C for 1h. The colour of reaction was developed by adding alkaline dinitrosalycilic (DNS) reagent as and the produced reducing sugars (as glucose) were measured according to Miller (1959). The absorbance was measured at 540nm against reagent blank. One unit of enzyme (CMCase) is defined as the amount of enzyme which released 1 micromole of reducing sugars, expressed as glucose per minute, under given condition.

β - Glucosidase Assay

β - Glucosidase or Salicin-ase enzyme activity was estimated by incubating 0.5 ml enzyme and 0.5 ml buffer (0.05 M citrate buffer pH 4.8) with 1 per cent salicin at 50°C (Mandels *et al*., 1976). Reducing sugars released in assay mixture were measured by dinitrosalicylic acid method (Miller, 1959). Enzyme activity was measured in terms of international units defined as the number of micromole of reducing sugar released per min per ml of test solution (measured as glucose).

Xylanase Assay

The enzyme assay has been measured according to Bailey *et al*. (1992) as follows: 1 per cent xylan (beech wood) was dissolved in 0.05M citare buffer (pH 4.8) and used as a substrate. 1mL of the culture supernatant (enzyme source, diluted if necessary) was added to 1ml substrate and the mixture was incubated for 15 min at 50°C. The released reducing sugars were determined by the method of Miller (1959). Xylanase unit is the amount of enzyme that liberates 1µ mole of reducing sugar (measured as xylose) per minute.

Lignin Peroxidase (LiP)

The enzyme assay was carried out in a standard reaction mixture consisted of 1 mL of 125mM 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 (Tien and Kirk, 1984).

Manganese Peroxidase (MnP)

The reaction mixtures included 0.1 mM $MnSO_4$, 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 per cent NaOH. The absorbance of the solution was measured at 610nm. The activity was expressed as the increase in A610 per minute per milliliter (Gold and Glen, 1988).

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 per cent tannic acid and incubated at 35°C (Bourbonnais and Paice, 1990).

Statistical Analysis

The data which are obtained from all the experiments were subjected to the statistical analysis to evaluate effects of treatments. Analysis was carried out by completely randomized design (CRD) using software WASP-2 tool (Duncan, 1995).

Results and Discussion

Isolation of Cellulolytic and Lignolytic Cultures

Based on different colony characteristics, total of 173 cultures including bacteria, fungi and actinobacteria were isolated from varied sources *viz*., paddy straw, degraded wood, termite midgut, cow dung, forest soil, paddy field soil, tree barks, compost and vermicompost with different types of bacterial colonies and distinct morphologies. All the isolates were subsequently purified and preserved (Table 1).

Primary Screening of Isolates for Cellulolytic and Ligninolytic Enzyme

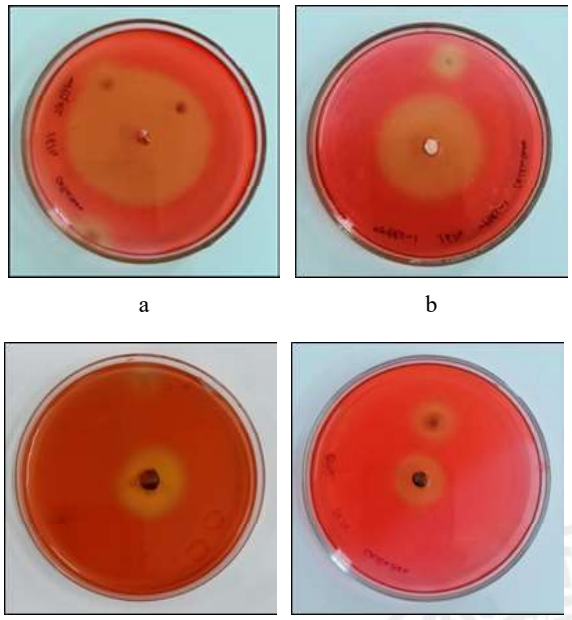
Qualitative Cellulase Enzyme Assay

All the 173 cultures were screened for their cellulase production by growing them on agar plates containing carboxymethyl cellulose (CMC) 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 173 isolates, 78 isolates showed the zone of clearance on the agar plate. Among the 78 isolates 32 fungi, 29 bacteria, and 17 actinobacteria were tested positive for cellulase assay (Plate 1). The isolation work was carried out to obtain efficient strains of greater hydrolytic power for degradation, which was checked qualitatively, where all the strains showed complete degradation of the substrate by forming clear zones after incubation, which shows that the microorganisms expressed the capability to produce complex of enzymes necessary for degradation of cellulose. The results were found to be in conformation with the findings of Nirmalasari *et al*. (2022). They identified

Table 1

Sampling sites, source and number of cultures isolated from different sites

Place	Source	GPS Location	Number of cultures isolated			
			Bacteria	Actino bacteria	Fungi	Total
GKVK	Rotten wood	13°04’48.4"N 77°34’52.9"E	6	3	5	14
	Swamp	13°04’45.3"N 77°34’53.2"E	4	3	6	13
	Compost	13°04’45.6"N 77°34’11.2"E	7	4	7	18
	Vermicompost	13°05’10.7"N 77°34’29.4"E	6	3	5	14
	Termite midgut	13°04’31.2"N 77°34’53.3"E	2	0	3	5
Siruguppa	FYM	15°38’48.6"N 76°52’26.5"E	11	6	5	22
	Paddy field soil	15°38’08.6"N 76°51’08.1"E	6	4	3	13
	Paddy field bunds	15°38’32.3"N 76°51’43.3"E	4	2	4	10
	Cow dung	15°38’50.9"N 76°52’19.3"E	7	4	3	14
	Paddy straw heap	15°38’47.3"N 76°52’26.0"E	4	0	1	5
KCDC	City compost		8	1	4	13
	Processed solid waste	12°53’38.4"N 77°38’59.2"E	3	2	2	7
	Processed straw waste		4	3	4	11
Ujire	Rotten wood	13°06’56.3"N 75°22’58.9"E	5	2	1	8
	Forest soil	13°07’01.4"N 75°23’10.0"E	3	1	2	6



a b

c d

Plate 1 : Qualitative cellulase enzyme assay, a- UASFW, b- UASFK2, c- UASBW1 and d- UASAP2

two types of potential fungi with higher potential index both ligninolytic and cellulolytic, including strain fungal CSF#5 and CSF#15 from the cocoa cropping pattern and were screened for their production of cellulase by using the CMC plate assay showing higher clear zone on CMC plate and forwarded for quantitative screening.

Qualitative Lignolytic Assay

Screening of lignolytic microorganisms were done using MSM agar media with alkali lignin (L) as their sole carbon source. All the 78 cellulolytic isolates obtained were screened for lignolytic activity. About 45 isolates which showed better growth in alkali lignin media indicates positive for lignin degradation. Similar results were found by Seesatat *et al*. (2021) reported the isolation of ligninolytic microbes from the forest soils. 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 and had greater efficiency of lignin degradation.

Secondary Screening of Isolates

Based on the cellulolytic and lignolytic activities, 45 cultures were selected for quantitative estimation of enzymatic assay.

Enzyme Assays

Cellulase enzymes like FPase, CMCase and β-glucosidase assays were performed to understand the cellulolytic activity of these isolates. These cellulase activity levels reflect the ability of the isolates to be actively involved in the saccharification process of the delignified or cellulose substrates. All fungi showed significant cellulolytic and lignolytic activity. Among 19 fungal isolates, UASFW isolate exhibited maximum activity in cellulase production and lignolytic enzymes, FP-ase (19.826 U/mL), CMCase (232.42 U/mL), β-D glucosidase (357.66 U/mL), Xylanase (2.713 U/mL), Laccase (547.23 U/mL), Mnp (19.83 U/mL) and Lip (1.25 U/mL) followed by isolate UASFK2 (Fig. 1). Among 17 bacterial isolates UASBW1 isolate exhibited maximum activity in cellulase production FP-ase (8.032 U/mL), CMCase (218.86 U/mL), β-D glucosidase (32.71 U/mL), Xylanase (0.832 U/mL), Laccase (0.42 U/mL), Mnp (8.32 U/mL) and Lip (13.87 U/mL) followed by isolate UASBW2 (Fig. 2). Comparing 9 actinobacterial isolates, the UASAP2 isolate result revealed a higher amount of cellulase and lignolytic enzymes production, with FP-ase (2.832 U/mL), CMCase (83.24 U/mL), β–D glucosidase (6.52 U/mL), Xylanase (0.125 U/mL), Laccase (0.069 U/mL), Mnp (0.142 U/mL) and Lip (0.119 U/mL) (Fig. 3). Overall cellulase production was higher in

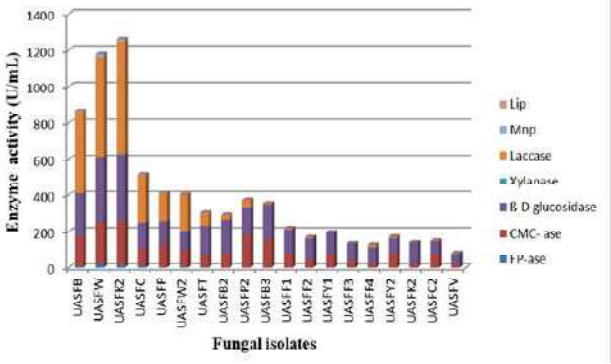


Fig. 1 : Lignocellulolytic enzyme activity of fungal isolates

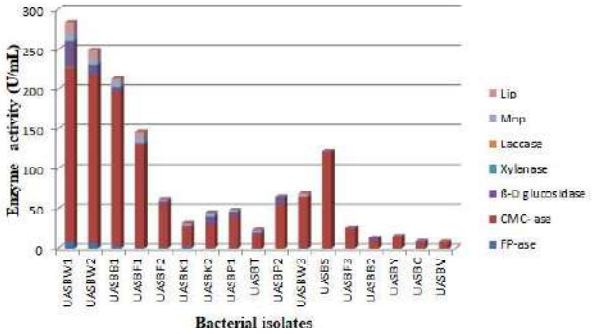


Fig. 2: Lignocellulolytic enzyme activity of bacterial isolates

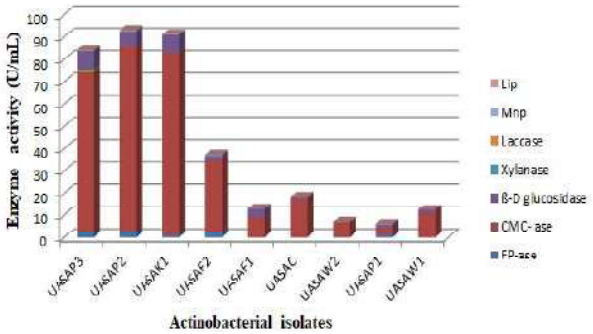


Fig 3: Lignocellulolytic enzyme activity of actinobacterial isolates

fungi compared to bacteria and actinobacteria. The quantity Laccase production by bacteria was not as good as fungi whereas, Lip and Mnp activity was better in bacteria and actinobateria compared to fungi.

Zhang *et al*. (2021) screened 36 isolates for their cellulolytic and ligninolytic activity, Bacterial isolate A10 showed the maximum units of enzyme activity in CMCase, Avicelase, laccase, Mnp and Lip (210.86 U/mL, 24.82 U/mL, 0.29 U/mL, 5.47 U/mL and 13.98 U/mL, respectively). Fungal isolate HJ exhibited the highest activity in CMCase, Avicelase, β-glucosidase, Laccase and Mnp (92.96 U/mL, 81.88 U/mL, 120.02 U/mL, 802.78 U/mL and 5.81 U/mL, respectively. Prasad *et al*. (2014) isolated 10 cellulolytic actinomycete strains and analysed the cellulolytic potential of the selected strains which showed that the strains CD-3 (0.023 IU/mL CMCase unit and 0.009 IU/mL FP-ase 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. Illuri *et al*. (2021) characterised lignolytic enzymes from various fungi, where laccase enzyme production ranged from 305.80 to 376.85 IU/mg, purified lignin peroxidase from 258.51 to 336.95 IU/mg, and purified manganese peroxidase from 253.45 to 529.34 IU/mg.

In the present study, the lignocellulolytic microorganisms were isolated from the samples collected from different ecosystems. Based on the qualitative and quantitative screening of the isolates for lignocellulolytic enzyme activity, it is concluded that four cultures *i.e*., two from fungi (UASFW and UASFK2), one bacteria (UASBW1) and one actinobacteria (UASAP2) were selected as the effective cellulose and lignin degrading microbes which produced all the lignocellulolytic enzymes. Microorganisms with a maximum lignocellulolytic potency could accelerate composting process which in turn could increase degradation rate. These cultures can be efficiently used for preparation of microbial consortium for degradation of agricultural residues.

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