Int.J.Curr.Microbiol.App.Sci (2017) *6*(12): 3808-3816

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 6 Number 12 (2017) pp. 3808-3816**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2017.612.438

Exploring Microbes for their Cellulolytic and Lignolytic Enzyme Activity for Manure Preparation

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Keywords

Biodegradation, *Bacillus*, Cellulase, Laccase, LiP.

Article Info

Accepted:
28 October 2017
Available Online:
10 December 2017

A B S T R A C T

In recent years, production of voluminous agricultural waste creates a major problem and is of environmental concern. Hence, there is great urge to adopt a reuse of agricultural waste. The objective of this study was to isolate cellulose and lignin degrading bacteria which are able to degrade agrowaste and convert agrowaste into green manure. Total of 103 bacteria were isolated. All these isolates were screened for their cellulase production by CMC agar plate assay. Out of which 51 bacterial isolates showed zone of clearance on CMC agar plates. 14 bacterial isolates which showed higher clearance zone on CMC agar plate were further selected for quantitative screening. Isolate 53 produced higher cellulase enzyme 0.636 U/ml. For screening of lignin degradation, ABTS plate assay was used for laccase enzyme. Out of all the 103 isolates, 10 showed positive result on ABTS agar plate. Among these 10 isolates, 4 isolate selected for further quantitative assay for Lignin Modifying Enzymes (LME) i.e., laccase, LiP and MnP. Isolate 72 showed higher laccase (453.6), LiP (95.0) and MnP (526.35) enzyme. Growth was also monitored upto 96 hrs of incubation during enzymatic studies. Two potential cellulolytic (isolate 53) and lignolytic (isolate 72) isolate were identified by 16 rDNA as *Bacillus licheniformis* C_1 and *Bacillus* sp. L_2, respectively.

Introduction

The lignocellulosic material of plants consists of the three main components, namely cellulose, hemicellulose and lignin. After cellulose, lignin is the second most abundant renewable biopolymer in nature. Lignin surrounds cellulose in the plant cell wall forming a matrix, which itself is resistant to degradation. Hence, degradation of plant materials which is ligno-cellulosic in nature is time consuming. The composting of agricultural residues rich in lignocellulose like paddy straw, sugarcane trash, banana pseudostem generally takes 180 days or more to obtain good and mature compost (Singh and Nain, 2014). The period of decomposition can be reduced by the use of efficient ligno-cellulolytic microorganisms. Bioaugmentation of cellulose and lignin degrading microorganisms reduce time and efficiently degrade crop residues such as straw, leaves, trash *etc.* (Gaur, 1999). However, the capacity of the microorganisms to assimilate organic matter depends on their ability to produce the enzymes needed for the degradation of substrate components *i. e.* cellulose, hemicellulose and lignin. Through the

synergistic action of microorganisms, complex organic compounds are degraded to smaller molecules, which can then be utilized by microbial cells. Till now, all the basics and applied research work has centered mostly on fungi. The stability of fungi is not good in practical treatment under extreme environmental and substrate conditions, such as higher pH, oxygen limitation, and high lignin concentrations (Crawford and Muralidharan, 2004). Bacteria are worthy of being studied for their cellulolytic and lignolytic potential due to their immense environmental adaptability and biochemical versatility. There are wide ranges of examples where bacteria like *Pseudomonas aeruginosa*, *Serretia marcescens*, *Nocardia*, *Arthrobacter*, *Micrococcus etc*. have been identified as ligno-cellulosic degrading microorganisms (Kalyani *et al.*, 2008). Cellulolytic microbes can convert cellulose in to soluble sugars by enzymatic hydrolysis. The complete degradation of cellulose is made by a cellulolytic enzyme system which contains three enzymes namely, β (1→4) endoglucanase, β (1 →4) exoglucanase and β-glucosidase (β-D-glucoside glucohydrolase or cellobiase). The endoglucanases act internally on the chain of cellulose cleaving β linked bond liberating non reducing end of cellulose chain. Finally, β-glucosidase completes the saccharification by splitting cellobiose and small cello oligosaccharides to glucose molecules (Prasad *et al.*, 2007). Similarly, lignolytic microbes produces the laccase, lignin peroxidase and manganese peroxidase enzymes which will also reduce the complex compounds in to simpler ones. The enzymes laccase, lignin peroxidase and manganese peroxidase are come under lignolytic enzyme systems. Laccase catalyzes the abstraction of one electron from the phenolic hydroxyl group to depolymerize lignin compounds. Laccase can also oxidize non-phenolic compounds when primary mediators like ABTS are present. It also produces Mn (III) chelates which allow wood-decaying enzymes to penetrate wood cell walls (Youn *et al.*, 1995). Lignin peroxidases are strong oxidants with high redox-potential that oxidize the major non- phenolic structures of lignin. Manganese peroxidase is a Mn dependent enzyme that catalyzes the oxidation of various phenolic substrates but not capable of oxidizing the more recalcitrant non-phenolic lignin (Abdel-Hamid *et al.*, 2013). Hence present study aims to isolate, screen and identify the bacteria which have the capability to produce cellulolytic and lignolytic enzymes.

Materials and Methods

Sample collection and isolation

Samples were collected from compost (NADEP) of organic farm, Navsari Agricultural University, Navsari, Gujarat, India, saline water and saline soil of Dandi farm, Gujarat, India, and from forest soil of Waghai during the year of December 2014 to December 2015. For isolation of bacteria, serial dilution method was used as describe APHA (1984). 1 g soil / 1 ml of water was serially diluted upto 10^{-7} dilution and 100 µl sample from each dilution was separately spread on nutrient agar plate. Based on different colony characteristics, total 103 different bacterial isolates were selected and purified further. Isolated pure colonies were stored on nutrient agar slants at 4 ^{o}C until the use. Sub-culturing was done at 15 days time interval.

Primary screening of isolates

Qualitative cellulase enzyme assay

For screening of cellulase producing microbes, Cellulose Basal Medium (CBM) ($C_4H_{12}N_2O_6$- 5, KH_2PO_4 -1, $MgSO_4.7H_2O$-0.5, Yeast Extract-0.1, $CaCl_2.2H_2O$ -0.001 in

g/litre distilled water) was used as described by Paterson and Bridge (1994). Isolated bacteria were streaked on CBM agar plates and incubated at 28 ^{o}C in the dark for 24 - 48 hrs. Clear zone on opaque agar media around the colonies indicate cellulose degradation. Zone of clearance was measured and zone diameter (mm) was noted down.

Qualitative laccase enzyme assay

Lignin degrading ability was measured using ABTS Agar Method. Laccase producing isolate convert ABTS (2, 2- Azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) to ABTS-azine a green color substance (Thurston, 1994). Isolates were streaked on ABTS agar and incubated at room temperature for 24 – 72 hrs. Change in color from blue to green was recorded and zone diameter was measured.

Secondary screening of isolates

Cellulase production

For quantitative cellulose assay, selected bacterial cultures were grown in nutrient broth for 18 hrs for inoculum. Cells were counted in Neubauer Counting Chamber under bright field microscope. 10^{8} cell/ml was inoculated in BHM broth supplemented with CMC as sole carbon source. Flasks were incubated at 28 ºC for 4 day. Growth kinetic was monitored by measuring optical density at 600 nm up to 96 hrs at 24 hrs of interval. Quantitative estimation of cellulase, laccase, lignin Peroxidase (LiP) and manganese Peroxidase (MnP) enzymes was performed with the following procedures from supernatant after removal of cells by centrifugation at 10,000 rpm for 10 min. All these enzyme activity was monitored up to 96 hrs at 24 hrs of interval. Control comprised uninoculated BHM supplemented with CMC.

Quantitative Cellulase Assay (DNSA Reagent Method)

The reducing sugar produced in the reaction mixture by cellulase enzyme was estimated by Dinitro-salicylic acid (DNS) method described by Miller (1959). One unit of enzyme activity is expressed as the amount of enzyme required to release 1 µM of glucose per minute under standard assay conditions.

Quantitative Laccase Enzyme Assay (ABTS Assay)

Laccase was assayed by monitoring the oxidation of 2, 2 azinobis (3-ethylbenz-thiazoline)-6 sulphonate (ABTS) by the enzyme extract (Wolfenden and Wilson, 1982) at pH 4.5 and 35^{o} C temperature. That result was then converted to µM of ABTS oxidized per minute to calculate the U/ml.

Quantitative Lignin Peroxidase Enzyme Assay

The LiP was estimated using demethylation of the methylene blue dye and measured on spectrophotometer as method described by Denise *et al.*, (1996). Briefly, 10 µl of enzyme sample (supernatant) was mixed with 1 ml sodium potassium tartarate (50mM, pH-4), 100 µl of H_2O_2 (0.1mM) and 32 µM methylene blue and incubated at room temperature for 1 hr. Optical density was measured at 650 nm. Decolorization of dye was estimated by subtracting the value from control and calculated as (A650 for control – A650 for test / A650 for control) x 100.

Quantitative Manganese Peroxidase assay

Manganese peroxidase activity was measured using H_2O_2 as substrate as method described by (Papinutti and Martinez, 2006). The result was expressed in U/ml.

Identification of bacterial isolate

Most potential two (one cellulolytic and one lignolytic) bacterial isolates were identified using 16S rDNA gene identification. DNA from both the isolates was extracted using CTAB method (Murray and Thomson, 1980). The bacterial 16S rRNA gene was amplified from the total genomic DNA using universal eubacteria specific primers *viz*., 27F and 1541R as described by Vyas and Murthy (2015) which yielded a product of approximately 1500 bp. The PCR conditions were: 35 cycles of 95 °C denaturation for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. Amplified PCR product was sequenced at 1st Base Laboratory, Singapore using Sanger's sequencing method. Sequence homology was explored using blast tool on NCBI website.

Results and Discussion

Isolation of cellulolytic and lignolytic bacteria

Based on different colony characteristics, total 103 different bacteria were isolated from different samples. All the isolates were subsequently purified and stored on nutrient agar slants. Isolates were given number from 1 – 103. Highest number of bacteria (42 isolate) were isolated from Dhaniya plot, Waghai followed by Kautukalaya plot, Waghai and least was from NADEP Compost, Organic farm, NAU, Navsari

Primary screening of isolates for cellulolytic and lignolytic enzyme

Qualitative cellulase enzyme assay

All the 103 isolated bacteria were screened for their cellulase production by growing them on agar which contained only cellulose as sole carbon source. Bacterial isolate able to produce cellulase, made zone of clearance near colony indicates cellulose degrading capabilities. Out of 103 bacteria, 50 isolates showed clear zone on agar plate (Table 1). Fourteen microbes (strain number 9, 14, 27, 48, 50, 53, 54, 69, 71, 72, 82, 67, 68 and 75) which showed higher zone of clearance and zone diameter were selected for further screening by quantitative assay for cellulase enzyme.

Qualitative laccase enzyme assay

For quantitative assay for laccase production, all the isolated bacteria were grown on LBM medium supplemented with 0.1 % ABTS. Isolate have ability to produce laccase change the blue color agar into green color indicating positive laccase producer. Out of 103 bacteria, 10 isolates showed green color formation. Among the 10 bacteria, 4 isolates (48, 67, 68 and 72) which showed prominent green color were selected for further quantitative analysis i.e. laccase, LiP and MnP.

Secondary screening of isolates

Quantitative enzyme assay

Isolates which showed higher clearance zone on agar were screened for quantitative cellulase enzyme production. Higher cellulase was produced by isolate 53 (0.636 U/ml) followed by isolate 9 (0.599 U/ml) after 48 hrs of incubation. Hence, the isolate 53 was selected as potential cellulolytic bacteria.

Quantitative laccase production

Laccase production was monitored after 24 hrs of incubation upto 96 hrs. Highest laccase enzyme was produced by isolate 72 followed by isolate 68 after 48 hrs of incubation (Fig 1a). Lease activity was observed in isolate 67.

Table.1 Temporal effect on cellulase production and growth of qualitatively selected microbes

Sr. No.	Sample No.	Cellulase enzyme activity (U/ml)				Growth of microbes (OD @ 600 nm			
		24 Hours	48 Hours	72 Hours	96 Hours	24 Hours	48 Hours	72 Hours	96 Hours
1	27	0.097	0.510	0.268	0.157	0.001	0.113	0.235	0.136
2	9	0.218	0.599	0.459	0.426	0.017	0.173	0.16	0.049
3	69	0.108	0.489	0.272	0.268	0.195	0.201	0.200	0.052
4	53	0.199	0.636	0.433	0.406	0.129	0.271	0.202	0.053
5	82	0.150	0.002	0.005	0.014	0.254	0.126	0.301	0.326
6	75	0.035	0.003	0.006	0.504	0.311	0.263	0.836	0.905
7	48	0.009	0.002	0.008	0.055	0.066	0.029	0.188	0.204
8	72	0.014	0.008	0.060	0.006	0.178	0.220	0.227	0.293
9	71	0.003	0.006	0.019	0.032	0.248	0.363	0.478	0.488
10	50	0.008	0.013	0.077	0.125	0.255	0.289	0.290	0.296
11	54	0.010	0.017	0.034	0.088	0.158	0.252	0.748	0.833
12	67	0.010	0.021	0.014	0.022	0.146	0.243	0.319	0.335
13	68	0.088	0.010	0.054	0.024	0.267	0.093	0.319	0.346
14	27	0.097	0.510	0.268	0.157	0.192	0.229	0.237	0.300

Fig.1 Temporal effect on growth and laccase production (a) LiP production (b) and MnP production by selected isolates

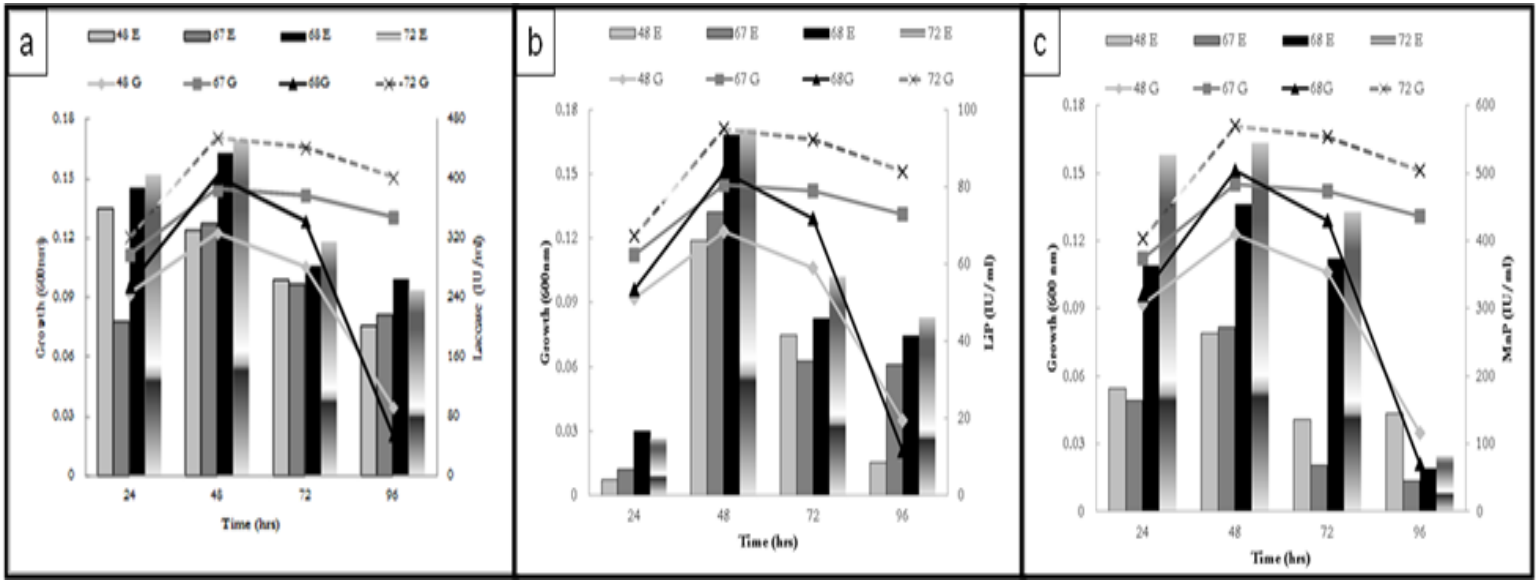
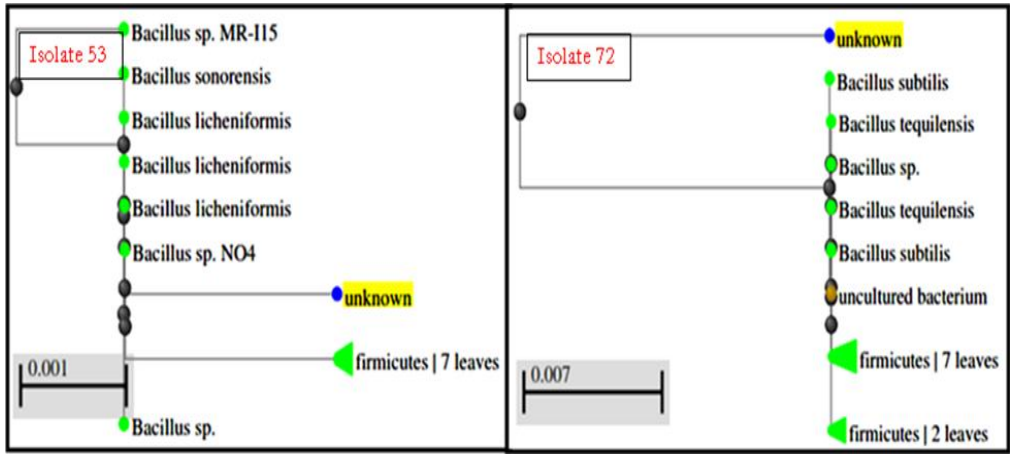


Fig.2 Phylogenetic tree of *Bacillus licheniformis* C1 and *Bacillus* sp. L2



Both growth and enzyme induction starts after 24 hrs of incubation and increase upto 72 hrs of incubation and thereafter both decreased.

Quantitative LiP production

LiP was estimated by demethylation of methylene blue dye. Isolate 72 produced higher enzyme (95 IU/ml) followed by isolate 68 after 48 hrs of incubation (Fig. 1b). Least activity was found in isolate 48. Maximum growth and enzyme production was observed after 48 hrs of incubation.

Quantitative MnP production

MnP enzyme was estimated using H_2O_2. Induction of MnP enzyme started after 24 hrs of incubation. Highest MnP was produced by isolate 72 (526.35 IU/ml) followed by isolate 68 (453.75 IU/ml) as shown in figure 1c. Least enzyme was produced by isolate 48. Isolate 68 and 72 showed maximum production of all the three enzymes. Among these two strains, isolate 72 was selected as efficient lignolytic microbe.

Identification of bacterial isolate

Based on primary and secondary screening for cellulolytic and LMEs, isolate 53 and isolate 72 were selected for cellulase and LME enzyme respectively. Genomic DNA of these two isolates was extracted using CTAB method and amplified using universal primer. PCR amplicon was of 1500 bp, which is typical product of universal primer. PCR product was sequenced at 1st Base Laboratory, Malaysia. Sequence homology was explored using blast tool on NCBI website. Isolate 53 showed 100 % homology with *Bacillus* licheniformis and isolate was named as *Bacillus* licheniformis C1 whereas, isolate 72 showed -- % homology with *Bacillus* spp and hence isolate 72 was named as *Bacillus* sp. L2. Sequence of the both the isolates were submitted to GenBank with accession number MF627836 and MF627837 respectively for isolate 53 and 72. Phylogenetic tree of both the isolates is shown in figure 2.

Currently huge amount of agricultural and industrial lignocellulosic wastes are generated and is accumulating in nature. Cellulose and lignin both are regarded as the most important renewable resource for bioconversion. Hydrolyzed cellulose has wide application in production of manure, ethanol, sweeteners etc. cellulosic matters are breakdown by cellulase, an inducible enzyme produced by microbes during their growth on cellulosic molecules (Lee and Koo, 2001). Many microbes have capabilities to degrade cellulose, however, only very few are potential to have cellulase which completely degrade cellulose. Lignin is the second most abundant constituent of the cell wall of vascular plants and it prevent cellulose from hydrolytic attack from saprophytic and pathogenic microbes. Its degradation represents a key step for carbon recycling in land ecosystems, as well as a central issue for industrial utilization of plant biomass. The lignin is a complex polymer of non- phenolic phenyl propanoid makes it more recalcitrant for biodegradation. A few microbes have ability to degrade this complex lignin by secretion of lignolytic enzymes. These are LMEs and generally are non-specific in nature having ability of benzene ring oxidation.

Amir (2011) had worked on isolation of bacteria from agricultural wastes and contaminated soil from different regions of Malaysia and screened for its cellulolytic and lignolytic properties. In present study bacteria from different sources among which higher number were isolated from coriander farming and least were isolated from NADEP compost. Total 103 bacteria were isolated for cellulase and LMEs production. Sheikhi *et al.,*

(2012) isolated 16 strains belonging to genus *Bacillus* as lignin degrading bacteria from soil and wastewater of a pulp and paper industry in Iran. Muhammad *et al.*, (2012) isolated and screened cellulolytic bacteria from soil and optimized the cellulase production and its enzyme activity.

Bai and co-worker (2012) have isolated cellulase producing *Bacillus subtilis* from cow dung. They have also identified isolate using 16S rDNA. Ladeira *et al.*, (2015) have isolated cellulase producing thermophilic *Bacillus* sp. SMIA-2.

Thus in present study total 103 bacteria were isolated from different soil and water sample and screened for cellulase and LMEs revealed that isolate 53 and isolate 72 were able to produce higher amount of cellulase and LMEs respectively. Both the isolates were identified by 16S rDNA and showed homology with *Bacillus* spp. Hence isolate 52 and 72 were designated as *Bacillus licheniformis* C1 and *Bacillus* sp. L2 respectively. Both the isolate can effectively used for the degradation of lignocellulosic waste degradation. However the detailed study is further required.

Acknowledgement

Authors are thankful to Department of Science and Technology (DST), Government of India for providing INSPIRE (Innovation in Science Pursuit for Inspired Research) fellowship as financial support for conducting this research work.

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How to cite this article:

Neethu T. Madhavan, K.G. Patel, Trupti K. Vyas and Sree Ganesh. 2017. Exploring Microbes for their Cellulolytic and Lignolytic Enzyme Activity for Manure Preparation. *Int.J.Curr.Microbiol.App.Sci.* 6(12): 3808-3816. doi: https://doi.org/10.20546/ijcmas.2017.612.438