Das et al. | Article 7763
J Pure Appl Microbiol. 2023;17(1):609-626. doi: 10.22207/JPAM.17.1.59
Received: 13 April 2022 | Accepted: 17 January 2023
Published Online: 03 March 2023

P-ISSN: 0973-7510; E-ISSN: 2581-690X

RESEARCH ARTICLE OPEN ACCESS

Isolation and Screening of Dye Degrading Lignocellulolytic Bacteria from Sundarban Mangrove Ecosystem, West Bengal, India

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Abstract

The present research work was carried out on isolating lignocellulolytic enzyme-producing natural bacterial isolates of the Sundarban Mangrove ecosystem in West Bengal, India. Multiple bacterial isolations and qualitative as well as quantitative screening of the isolates was conducted by selective media plates and dye decolorization assay. Growth characterization of the top three isolates, namely GD1, GD2, and GD3, was determined in both nutrient broth and selective media. GD1 showed laccase activity in ABTS plate and decolorized congo red, malachite green, and methylene blue by 64.82%, 47.69%, and 33.33%, respectively, which is the inherent property of laccase. Along with the maximum laccase activity (9.72U/L), it also showed a little amount of cellulase (5.8U/ml) and pectinase (0.55U/ml) activity in the enzyme assay. GD2 showed maximum cellulase activity (6.56U/ml) with only 3.3% degradation of congo red and 39.43% degradation of malachite green. Like GD1, GD2 also bears 5.6U/L laccase and 0.12U/ml pectinase enzyme activity. The last isolate, GD3 specialized in pectinase production and having 4.4U/ml enzyme activity, degraded congo red by 66.75% and malachite green by 54.26%. All isolates showed activity between 30° and 37°C, and pH ranges from 4.5 to 7. The outcomes of this research will be useful in the fiber industry like jute or banana, dye industry, pulp-paper industry, and textile industry for waste remediation, recycling, and fiber modification.

Keywords: Laccase, Cellulase, Pectinase, Dye-decolorization, Enzyme Activity

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Citation: Das S, Sinha N, Sen M, Ghosh D. Isolation and Screening of Dye Degrading Lignocellulolytic Bacteria from Sundarban Mangrove Ecosystem, West Bengal, India. *J Pure Appl Microbiol.* 2023;17(1):609-626. doi: 10.22207/JPAM.17.1.59

INTRODUCTION

Lignocellulosic biomass is a plant waste material that has become a highly influencing matter nowadays to the scientific community as it is a renewable energy source and can be converted to value-added products. But its recalcitrant structure protects it from degradation, recycling, and conversion to high-impact materials. It is composed of lignin, pectin, cellulose, hemicellulose, etc. Among them, lignin is a highly complex polymer of polyphenolic compounds such as syringyl (S), guaiacyl (G), and p-hydroxyphenyl (H) units which are present in 66:32:2 ratios.[1] Different industrial fibers like jute, banana, hemp and kenaf are lignocellulosic materials in which lignin composition ranges from 13.3 to 15%.[1-3] Different lignocellulosic fiber contains a high amount of lignin that restricts its use in industry as the percentage of lignin in lignocellulosic fiber is inversely proportional with fiber fineness.[4] Photo yellowing is another problem in lignocellulosic jute fibers that occurs due to the reaction between UV radiation and an α-carboxyl group of lignin[2] that lowers the quality of fabrics and apparel produced in the textile industry. In the pulp-paper industry, the bleaching of wood is a major source of pollution. It involves the release of toxic chemicals into the environment.[5] So, along with the fiber industry, different other industries, like the pulp-paper, dye, and textile industries, generate a high amount of lignocellulosic waste effluents that have a negative impact on the environment starting from agriculture and irrigation to pisciculture[6]. Hence, these multi-diverse industries need a way to generate low-lignin-based fiber for quality textile products as well as dye detoxification by dye decolorization, effluent recycling for reduction of organic load, and fermentation of de-lignified sugars for biofuel generation. Chemical and physicochemical treatments like organo-solvent, oxidative delignification, wet oxidation, alkaline pretreatment, microwave heating with catalyst technology, natural dyes treatment, ionic liquids, acidic pretreatment, etc., can be used for delignification, but that processes are neither sustainable nor economically feasible.[7-9] Hence biological root is much better, having few limitations.[10] The industrial utilization of fungal enzymes is very much restricted in accordance with its genetic manipulations, protein expression level, and lower stability under high pH conditions[11]. On the contrary, bacterial enzyme system is highly productive in respect of time and non-toxic.[12] The lignin-degrading ability of bacteria is highly commanding than fungi due to its ability to better adapt to anaerobic conditions[10] and biochemical versatility[13]. Delignification is carried out by a series of ligninolytic enzymes of bacterial and fungal origin, namely laccase, lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase (VP).[14] The MnP oxidizes the phenolic compound, whereas LiP and laccase oxidize the non-phenolic part of lignin, respectively.[15-16] Laccase-mediator technology is highly effective for converting complex micropollutants into less toxic molecules in an eco-friendly manner. It can also modify the structures of different azo dyes by breaking their chromophoric group. As a result, industrial effluents treated with this enzyme show low toxicity and can be used in the seed germination process.[17-21] Other biocatalysts like pectinase and cellulase, also known as carbohydrase, are responsible for de-cementing the lignocellulosic biomass (LCB) and the breakdown of the released polysaccharide into simple glucose monomers respectively,[22] that can be fermented further for the production of present generation biofuel. The Mangrove ecosystem is a high brine area with unique properties, like fluctuations in oxic/anoxic conditions, pH, and tidal, which always stress the microbiome there. Mangrove plant roots and Mangrove seawater are potential sources for a diverse group of highly productive lignocellulolytic microbial communities capable of degrading lignocellulosic biomass as it is rich with plenty of decayed plant materials. It is also a suitable environment for cellulase exploration as input of the lignocellulosic carbon is continuously going on in the form of litter that acts as a substrate for degradation by microbes.[23] Isolation and screening of lignocellulolytic microbial cell factories from Mangrove ecosystems are scattered and bacterial breakdown of lignocellulosic biomass from Mangrove ecosystems is not well characterized. To this end, the present study was carried out using bacterial samples from different locations of the Sundarban Mangrove ecosystem, West Bengal, India, to identify their abilities for the breakdown of lignocellulosic biomass as well as dye

decolorization for waste remediation and recycling that can be a potential workhorse in future for different industries for sustainable productivity with pollution-free nontoxic ecosystem.[24-26]

MATERIALS AND METHODS

Sample Collection

The soil and liquid samples were collected from the Dobanki (Latitude DMS: 21°59'19.99"N, Longitude DMS: 88°45'18.72"E), Pakhiraloy (Latitude DMS: 22°8'30.68"N, Longitude DMS: 88°50'0.61"E) Bidyamandir (Latitude DMS: 21.9889° N, Longitude DMS: 88.7552°E) of Sundarban Mangrove forest in West Bengal, India. The samples were collected in sterile zipper packets and sample bottles and transported to the laboratory within 24 hrs of collection. Then samples were stored at 4°C for further analysis.

Chemicals

The chemicals that were used to conduct the research were as follows:

Carboxymethyl cellulose, Sodium nitrate, Dipotassium phosphate, Potassium chloride, Magnesium sulfate, Ferrous sulfate, Yeast extract, Agar, Different dyes like Malachite green, Congo red, Methylene blue,Glucose, Malt extract, Peptone, Wheat bran, $(NH_4)_2SO_4$, K_2HPO_4, KH_2PO_4, DNS (3,5 dinitro salicylic acid), ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)), Galacturonic acid, Citrous Pectin, Commercial pure laccase, Commercial pure cellulase, Commercial pure pectinase. All chemicals were purchased from Sigma-Aldrich, Loba Chemie, Himedia, Thermofisher scientific India, etc.

Instruments

Light microscope (400X, 1000x magnification in Magnus MLXi Plus, Magcam DC3), UV-Vis spectrophotometer (SHIMADZU, Model No: UV-1800). 4°C centrifuge (10000 rpm.) CM-8 PLUS, REMI, Water bath (Sarada laboratories, India), Laminar flow (HEPA filter 0.3-micron, area: 3×2×2 ft., Sarada laboratories, India), Incubator (Sarada laboratories, India), Vacuum freeze-drying apparatus (EYELA, FDU-1200), Perkin-Elmer Frontier FTIR spectrophotometer.

Preparation of dinitro salicylic acid (DNS) solution

The formulation of the DNS solution was carried out by the process stated by Kriger et al.[27]

Isolation and screening of lignocellulolytic enzyme-producing bacteria

The isolation of lignocellulolytic indigenous bacteria was carried out by serial dilution of soil and water sample. The Sample was serially diluted ($10^{-1}$to 10^{-9}). 0.1 ml from the dilution 10^{-3} were plated in Nutrient agar plates using the spread plate method and incubated at 37°C for 24 hours for bacterial counts. Colonies on the plates were counted manually.[28-30] Then, a single colony was plated by the streak plate method on different selective media at 30°C to 37°C for 24 to 48 hours of incubation. Some selective media for laccase, cellulase, and pectinase were used for the primary screening of the microorganisms able to degrade lignocellulosic components like lignin, cellulose, and pectin. Selective media were made by mixing the components properly, as stated by Pundi et al., Atri et al., and Tekere et al.[30-32] The growth medium was autoclaved and transferred to Petri plates. Inoculated the selective media with test microorganisms and incubated at 30°C to 37°C for 48 hours. Then the primarily screened bacterial isolates were preserved on the selective medium at 4°C.

Qualitative analysis of lignocellulolytic microorganisms

The natural isolates were then checked for the determination of the potentiality of lignocellulose breakdown by the plate assay.

Qualitative analysis of laccase-producing microorganisms

Two different processes determined the qualitative analysis of the laccase enzyme. In the first case, the microbial cultures were streaked on Luria Bertani agar plates supplemented with ABTS and incubated at 30°C-37°C for 24 hours. Laccase enzyme production was confirmed by green color formation on the ABTS-LB agar plates. The formation of intense green color was a positive sign of laccase. In the second case, after confirming the bacterial colony on the laccase selective plate, wells were made on the laccase

selective media containing 1% malachite green and congo red and methylene blue plate, and 100µl of selected isolates were poured on the wells and incubated for 48 hours of incubation at 37°C. The discoloration zone around the wells was a sign of a positive result. Only these isolates (GD1) were then taken for further study.[33]

Qualitative analysis of cellulase-producing microorganisms

After confirming the bacterial colony on the CMC-agar selective plate, two different strategies were applied. In the first case, wells were made on the CMC-agar selective plate, and 100µl of the selected isolate was poured into each well. After 48 hours of incubation at 37°C, the media-containing plate was poured with 1% w/v aqueous Congo red. After 15 minutes, the stain was poured off and washed on the agar surface with distilled water. Lastly, 1M NaCl was added for 15 mins and washed off. The discoloration zone around the wells was a sign of a positive result. Only these isolates (GD2) were then taken for further study. In the case of malachite green degradation, wells were made on the CMC-agar selective media containing a 1% malachite green plate, and 100µl of the selected isolate was poured on the well and incubated for 48 hours of incubation at 37°C. The discoloration zone around the wells was a sign of a positive result.[34]

Qualitative analysis of pectinase-producing microorganisms

After confirming the bacterial colony on the Pectinase selective plate, the same process was applied for Congo red degradation. The discoloration zone around the wells was a sign of a positive result. Only these isolates (GD3) were then taken for further study.[35]

Quantitative analysis of lignocellulolytic microorganisms

Primarily screened potential lignocellulolytic natural isolates GD1, GD2 and GD3 were taken for quantitative estimation by different dye-decolorization processes. The potentiality of the positive natural isolates was quantitatively measured by calculating the dye hydrolysis percentage.

Quantitative analysis of laccase-producing microorganisms by dye decolorization

1% Congo red, malachite green, and methylene blue was prepared. 20ml of sterile laccase selective liquid media were taken in 100ml conical. 200µl dye was mixed with the laccase selective medium, and 200µl of 24-hour culture (GD1) that provided positive results in the laccase plate assay was also added to the conical. After adding the culture, 2ml of the mixture was collected in 2ml sterile Eppendorf and centrifuged at 10000rpm for 8min. The supernatant was collected, and optical density was taken on 417nm, 617nm, and 668nm, respectively. The same process was continued every 2 hours up to 6 hours. In the spectrophotometer, selective media was taken as blank. The dye decolorization percentage was calculated every 2hours.[29]

Quantitative analysis of cellulase-producing microorganisms by dye decolorization

1% Congo red, malachite green, and methylene blue was prepared. 20ml of sterile CMC selective liquid media were taken in 100ml conical. 200µl dye was added to the selective medium, and 200µl of 24-hour culture that provided positive CMC-agar plate assay results were added to the conical. After adding the culture, 2ml of the mixture was collected in 2ml sterile Eppendorf and centrifuged at 10000rpm for 8min. The supernatant was collected, and optical density was taken on 417nm, 617nm, and 668nm, respectively. The same process was continued every 2 hours up to 6 hours. In the spectrophotometer, selective media was taken as blank. The dye decolorization percentage was calculated every 2 hours.[36]

Quantitative analysis of pectinase-producing microorganisms by dye decolorization

1% Congo red, malachite green, and methylene blue was prepared. 20ml of sterile Pectin liquid media were taken in 100ml conical. 200µl of 1% dye was poured into the selective medium, and 200µl of 24-hour culture that provided positive results in the Pectin-agar plate assay was added to the conical. After adding the culture, 2ml of the mixture was collected in 2ml sterile Eppendorf and centrifuged at 10000rpm for 8min. The supernatant was collected, and

optical density was taken at 417nm, 617nm, and 668nm, respectively. The same process was continued every 2 hours up to 6 hours. In the spectrophotometer, selective media was taken as blank. The dye decolorization percentage was calculated every 2hours.[35]

Growth curve determination of selected microbial isolates

After the screening process, most potential bacterial (higher enzyme diffusion rate in selective plate) growth characteristics were determined by measuring the bacterial growth in the nutrient broth medium as well as in the respective selective medium at a specific time interval with the help of a spectrophotometer by measuring the absorbance at 600nm for finding their exponential phase that we can use for our further research.

Enzyme Assay

Substrate specificity

To determine the presence of multiple lignocellulolytic enzyme activities in the GD1, GD2, and GD3 isolates, the isolates were incubated with all three specific substrates like ABTS, CMC, and pure pectin-containing media that were then incubated overnight at 37°C. Then all three-enzyme activity (laccase, cellulase, and pectinase) was measured by following enzyme assay methods.

Laccase assay

Laccase enzyme activity was measured by observing ABTS oxidation. ABTS is a nonphenolic dye oxidized by laccase to the more stable cation radical. The cation radicals generated after oxidation are responsible for the blue-green color directly correlated with the enzyme activity, and it can be read at 420 nm (ε^{420}=3.6 × 10^4 M^{-1}·cm^{-1}) in a spectrophotometer against a proper blank. The assay reaction mixture contained 0.2ml of 0.5 mM ABTS, 1.7ml of 0.1 M sodium acetate buffer (pH 4.5), and 0.1ml of the crude enzyme incubated for 10 min. One unit of the enzyme is defined as the amount of laccase that oxidized 1 μmol of ABTS substrate per min.[37]

Laccase enzyme activity (U/L) = (ΔE×Vt)×10^6 / (t×ε×d×Vs)

ΔE= Change in the extinction of light [min^{-1}] at 420 nm,

ε = Molar absorption coefficient of ABTS [M^{-1} cm^{-1}]

d = Thickness [cm] of the cuvette

V_t = Total volume measured

V_s = volume of the enzyme added.

t = Reaction time (min)

Cellulase assay

Total cellulase activity was measured by determining the amount of released reducing sugar from amorphous cellulose. The enzyme activity was measured according to the recommended methods of the International Union of Pure and Applied Chemistry (IUPAC) Commission on biotechnology. CMCase assay mixture contained 0.1 mL of crude enzyme and 0.2 mL of 1% CMC in 1.7 ml 50 mM citric acid buffer (pH 4.8). It was then incubated at 50°C in a water bath for 60 min and closed the reaction by adding 3 mL of 3,5-dinitrosalicylic acid (DNS). Reducing sugar levels in the supernatant was determined at 540 nm. One unit of enzymatic activity can be defined as the total amount of enzyme that can release 1 µmol of reducing sugars (glucose) per mL per minute.[34]

Cellulase enzyme activity (U/ml) = W×1000/V×t×M

Here,

W - Amount of released glucose

M - Molecular weight of the glucose

V - Volume of the sample

t - Reaction time

Pectinase assay

To determine the pectin enzyme activity,2 mL cultures were centrifuged at 10,000 rpm for 8 min. The supernatant was represented as crude enzyme extract. 1% (w/v) pectin was used as substrate. 1.7ml of 0.1 M (pH 7) phosphate buffer, 200 µL of the substrate, and 100 µL of enzyme extract were mixed and kept in a water bath for 10min at 50°C. The temperature of the mixture was then lowered to room temperature, and 2 ml of DNS reagent was added. The mixture was then kept in a boiling hot water bath for a minimum of 5 min. After that, the optical density of the solution mixture was measured at 540

nm using a spectrophotometer. Both enzyme blank and substrate blank were measured for the quantitative analysis of pectinase. One unit of enzyme activity (U) can be defined as the enzyme required releasing 1 µmol of the reducing sugars per minute.[38]

Pectinase enzyme activity (U/mL) = (µg galacturonic acid released × V) / v × 194.1 × t

Here V= Total volume of solution

v= Volume of the crude enzyme

194.1 is the molecular weight of galacturonic acid

t= Reaction time in minutes.

UV and FTIR analysis of dye decolorization

The degradation and changes in chemical bonds of CR, MB, and MG dyes were studied and observed with UV-Visible, and Fourier transform infrared spectroscopy (FTIR). Samples were withdrawn after 6hrs. of incubation in nutrient broth medium and centrifuged at 12,000 rpm for 10 min to remove all bacterial cells. Then, with the help of a vacuum freeze-drying apparatus (EYELA, FDU-1200), some supernatant was dried to constant weight for FTIR analysis. The remaining supernatant was used to analyze the UV spectrum analysis. The dried powder samples formed by lyophilization were used for FTIR analysis. FTIR and UV spectra were measured in 4000–400-cm^{-1} region and 400-190nm region by Perkin-Elmer Frontier FTIR spectrometer and UV-Vis spectrophotometer (SHIMADZU-UV-1800), respectively. The untreated dyes without bacteria were used as the controls in the UV and FTIR analysis. The degraded and undegraded dye samples were properly mixed with KBr to form a pellet which was then fixed in the sample holder for FTIR analysis.[39]

Phylogenetic study considering bootstrap and outgroup applications

Laccase, Cellulase, and Pectinase enzymes FASTA sequences for representative bacteria were retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/). Multiple sequence alignment was done for each enzyme group sequence by the Muscle algorithm, and phylogenetic analysis was carried out by MEGA 11 software. The tree was obtained by the Neighbour-Joining method. A particular out-group was chosen from the fungal origin accordingly for each enzyme. The root was placed based on the out-group. The consistency of the phylogenetic tree was analyzed by the bootstrap method.[40-41]

RESULTS AND DISCUSSION

Isolation and qualitative Screening of Lignocellulolytic Isolates

This present research is an endeavour to analyze potential microbial systems for their selective lignocellulolytic activity. In the present research, we have isolated morphologically different natural pure cultures from soil and water

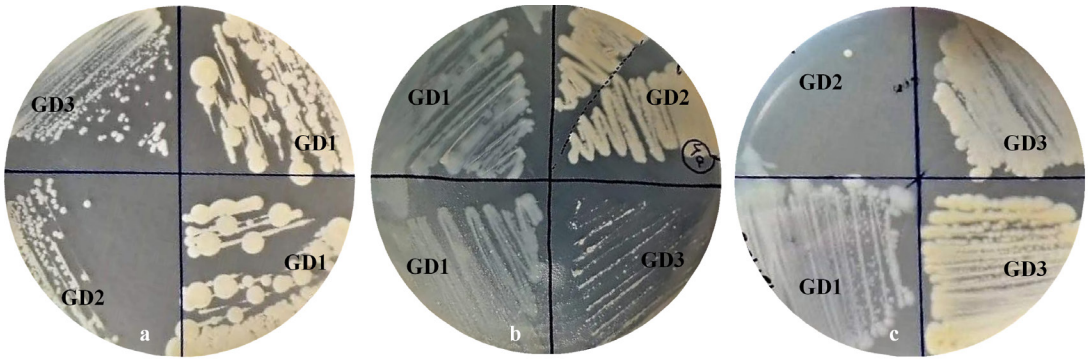

Figure 1. Isolation of Lignocellulolytic Mangrove strains on selective medium. (a). Plate (a) is a laccase selective media plate, where GD1 actively grows, but the growth of GD3 and 4 was relatively slow. (b). Plate (b) is a Cellulase selective plate (CMC-agar plate), where cellulose breaking bacterial system grew fast, GD2, but GD1 and GD3 showed relatively slow growth. (c). Plate (c) is a pectinase selective media plate where GD3 showed the highest pectinase production and GD2 showed the lowest

samples of the various location of Sundarban, namely Dobanki, Pakhiraloy, and Bidyamandir, on selective media plates (Figure 1). All isolates were qualitatively screened depending on the clear zone formation on the selective media supplemented with the suitable specific indicators (plate assay), indicating that isolates have ligninolytic, cellulolytic, and pectinolytic activity.[42] The highest zone-forming isolates were further studied (GD1, GD2, and GD3). Laccase-positive isolates were first identified in the LB-agar plate by oxidation of ABTS forming green color (Figure 2a) and also identified by the decolorization of malachite green and congo red in laccase selective plates[43] (Figure 2b, 2c). Supplements with specific phenolic substrates indicate extracellular secretion of ligninolytic enzymes which was proven in different research.[44-46] All the cellulolytic isolates were tested for cellulase activity by congo red-CMC agar and malachite green CMC-agar plate assay that produced non-homogenous clear zones (CMC clearance) and the decolorized zones of congo red CMC-agar (Figure 3b) and malachite green CMC-agar (Figure 3a) demonstrated the cellulose-degrading capacity[47] and dye decolorization ability. Cellulase plays a crucial role in the breakdown of lignocellulosic biomass to release the fermentable sugar monomers that are then used in enzymatic saccharification for the production of biofuel. Hence, cellulase enzyme utilization is very critical.[48] For pectinase plate assay that produced non-homogenous clear halo zones demonstrated

Figure 2. Laccase activity by GD1. (2a) This figure depicts the oxidation of ABTS by the laccase activity of GD1 in the LB-agar ABTS plate, producing green color. (2b) This figure depicts the homogenous diffusion of laccase and malachite green breakdown with a halo zone formation. (2c) This figure Depicts the non-homogenous diffusion of laccase in the Congo red-selective plate and the breakdown of Congo red by laccase with the formation of a halo zone

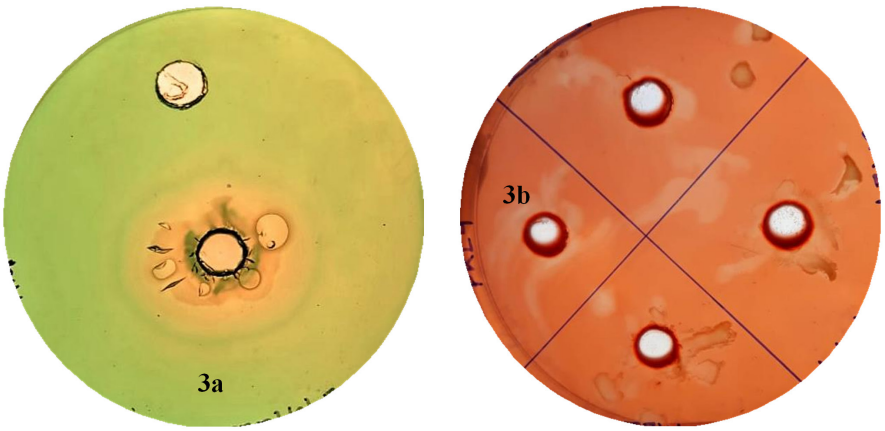

Figure 3. Cellulase activity by GD2. (3a) This figure depicts CMC and malachite green breakdown by cellulase-producing GD2 that results in a halo zone formation. (3b) This figure depicts the non-homogenous diffusion of cellulase in the Congo red-CMC-agar plate and the breakdown of Congo red by cellulase

the capability of the isolate to degrade pectin and also dye degrading ability[38] (Figure 4). In the present research, it was observed that the isolated bacterial isolates showed a high level of lignocellulolytic activity and hence they were, therefore, considered key potential candidates for further research that will have a significant contribution to the industrial sectors.[49]

Growth curve analysis

After the screening process, the selected lignocellulolytic bacterial isolates were then analyzed for their growth curve in both nutrient broth media (Figure 5) as well as respective selective media (Figure 5). It was observed that in both media all isolates showed similar types of growth characteristics. All isolates showed the start of their exponential phase between 3 hours to 4 hours of incubation and reached their death phase approximately after 24 hours of incubation.

Figure 4. Pectinase activity by GD3 in Congo red Pectin-agar selective media and the breakdown of Congo red by non-homogenously diffused pectinase of GD3, forming halo zone

Quantitative analysis by dye decolorization

Three different dyes namely congo-red, malachite green, and methylene blue were used for the quantitative determination of the dye decolorization potentiality of the isolates GD1, GD2, and GD3 (Figure 6). These dyes were degraded by laccase, cellulase, and pectinase in the respective selective medium. The dye breakdown rate was monitored every 2 hours, 4 hours, and 6 hours time intervals at specific wavelengths (Congo red-417nm, Malachite green-617nm, and Methylene blue-668nm). The decolorizing percentage of dyes by each isolate is listed in Table 1,2,3 and the decolorizing percentage of three different dyes by each isolateis compared graphically in Figure 7a and 7b. It is surmised that dye molecules are absorbed on microbial surfaces and then degradation takes place. Calculation of dye degradation percentage was carried out by the following formulae:

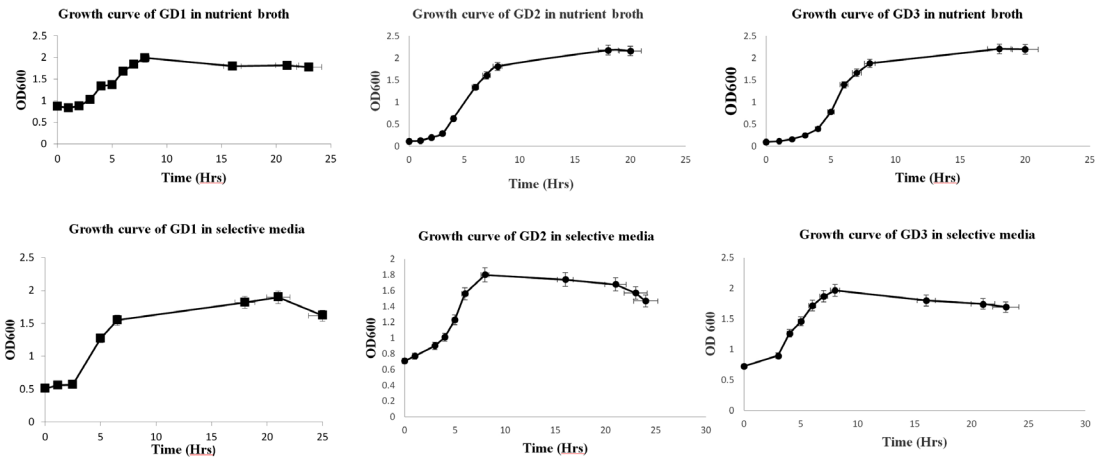

Figure 5. Growth curve determination of selected microbial strains in nutrient broth and selective media. Figure 5 depicts that all strains show approximately similar type of growth characteristics. Exponential phase for all three strains starts between 3hr to 4hrs and reached their death phase approximately after 24hrs of incubation.

Decolorization (%) = (Initial absorbance – Final absorbance)/ Initial absorbance × 100

Lignocellulolytic enzyme activity

Standard curve of glucose for the determination of cellulase enzyme activity

Five solutions having different concentrations likely 0.1 mg. mL^{-1}, 0.2 mg. mL^{-1}, 0.4 mg. mL^{-1}, 0.8 mg. mL^{-1}, and 1mg. mL^{-1} of glucose was measured in distilled water. Afterward, it had been incubated at 40°C for 10min. Then 2ml of each concentration was added to a test tube along with a 2ml DNS solution. Test tubes were then placed in a hot water bath with boiling water for 10 minutes and cooled. A blank sample was prepared (2ml of distilled water and 2ml DNS solution). Optical density was taken at 540 nm and a standard curve was prepared by plotting the absorbance value against concentration[50] (Figure 8).

Standard curve of galacturonic acid for determination of pectinase enzyme activity

Four solutions of 5ml volume have different concentrations, likely 1mg/mL, 0.5mg/mL, 0.25mg.mL^{-1}, and 0.125 mg.mL^{-1} of galacturonic acid was measured in distilled water and incubated the solutions at 40°C for 10min. Then each concentration of 5ml volume was added to a test tube along with a 5ml DNS solution. Test tubes were then placed in a hot water bath with boiling water for 10 minutes and cooled. A blank sample was prepared (5ml of distilled water and 5ml DNS solution). Optical density was taken at 540

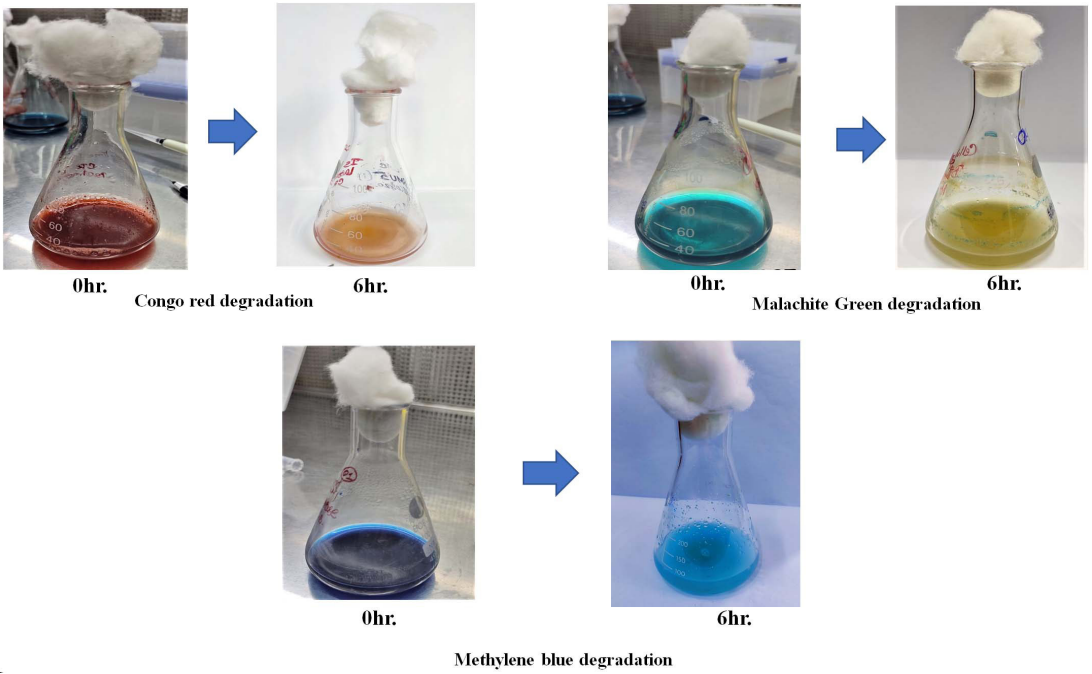

Figure 6. Dye decolorization by different lignocellulolytic enzymes produced by GD1, GD2 and GD3

Table 1. Different dye degradation by Mangrove isolate GD1

Isolate name	Name of the dye	λmax	Time required	Degradation %
GD1 (Isolate from Dobanki, Sundarban) (Latitude DMS: 21°59'19.99"N, Longitude DMS: 88°45'18.72"E)	Congo red	417	6hrs	64.82
	Malachite green	617	6hrs	47.69
	Methylene blue	668	6hrs	33.33

nm and a standard curve was prepared by plotting the absorbance value against concentration[51] (Figure 8).

Enzyme assay

From all the enzyme assays, it can be concluded that all three isolates contain lower to higher amounts of all three-enzyme activities: laccase, cellulase, pectinase, etc. It can be predicted from the results that these isolates possess genes responsible for this activity but are not properly controlled hence the expression level varies widely. The Comparison among three isolates for all three-enzyme activity has depicted in Figure 9.

UV and FTIR analysis of dye decolorization

UV–spectra (190–400 nm) and FTIR (4000–400-cm^{-1}) analysis were carried out for the analysis of dye decolorization of CR, MB, and MG dyes that were treated by the isolates GD1, GD2, and GD3 (Figure 10, 11). The major peaks for UV-spectra were found between 200-220 nm at 0 h. After 6h,it was observed that the maximum absorbance peak was shifted to a new wavelength or completely disappeared. The reduction in

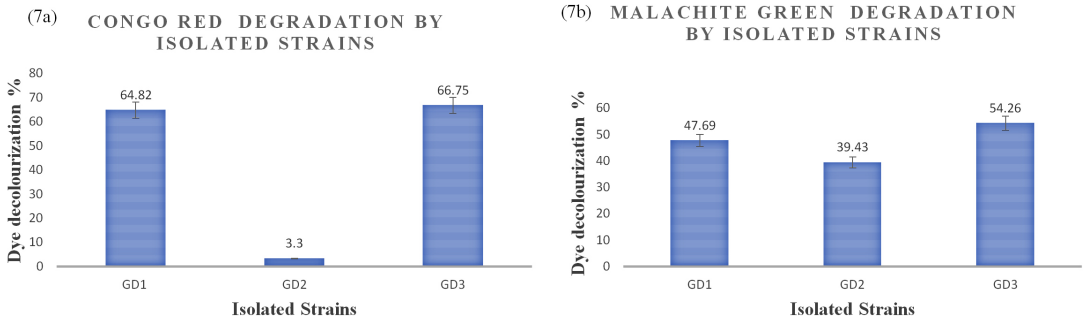

Figure 7. Comparison among GD1, GD2, and GD3 for dye decolorization efficiency. (7a) This figure depicts that strains GD1 and GD3 are highly capable of degrading congo red at 64.85% and 66.75%, respectively, but GD2 cannot properly degrade Congo red. (7b) In the case of malachite green degradation, all three strains are approximately equally capable

Table 2. Different dye degradation by Mangrove isolate GD2

Isolate name	Name of the dye	λmax	Time required	Degradation %
GD2 (Isolate from Bidyamandir, Sundarban) (Latitude DMS: 21.9889° N, Longitude DMS: 88.7552° E)	Congo red	417	6hrs	3.3
	Malachite green	617	6hrs	39.43
	Methylene blue	668	6hrs	Unable to degrade

Table 3. Different dye degradation by Mangrove isolate GD3

Isolate name	Name of the dye	λmax	Time required	Degradation %
GD3 (Isolate from Pakhiraloy, Sundarban) (Latitude DMS: 22°8'30.68"N, Longitude DMS: 88°50'0.61"E)	Congo red	417	6hrs	66.75
	Malachite green	617	6hrs	54.26
	Methylene blue	668	6hrs	Unable to degrade

absorbance peak area confirms the decolorization of CR, MB, and MG dyes by the biodegradation process, indicating the action of bacterial isolates and their dye metabolizing activity.[52]

Mono-substituted and para-disubstituted benzene rings were the most distinct peaks for CV at 0 h, supporting the peak between 1500-1800 cm^{-1} due to the C=C stretching of the benzene ring. Additionally, the spectrum displayed peaks between 1100-1200 cm^{-1} for C-N stretching vibrations and 2900-3000 cm^{-1} for C-H stretching of the asymmetric CH_3 group. The peak in the region between 2300 and 2400 cm^{-1} was due to the tertiary amine salt being stretched symmetrically and asymmetrically. At 1300-1400 cm^{-1}, an aromatic tertiary amine's C-N stretch peak was noted. Following biodegradation by GD1, GD2, and GD3, a degradation of the aromatic ring was highlighted in the range of 1600-1700 cm^{-1}. This peak can be attributed to the C=C stretch.[53]

In the case of methylene blue, the Untreated dye's FTIR spectrum displayed distinct peaks between 3400-3500, 1400-1500, and 1100-1200 cm^{-1}, which were associated with NH, N=NO, and CS stretching, respectively. The presence of the CH_2 group and COH bending were indicated by the peaks between 800-900 and 600-700 cm^{-1}, respectively. While the FTIR spectrum of treated methylene blue displayed peaks between 3500-3600 cm^{-1} for NH and OH stretching, 1100-1200 cm^{-1} for CS stretching, and 3399 cm^{-1} for OH. C-N and OH bending was represented by peaks between 1400-1500 cm^{-1}, and 600-700 cm^{-1}, respectively. The peak at approximately 3000 cm^{-1} represented that N=N=N antisymmetric stretching was present. The findings showed that numerous

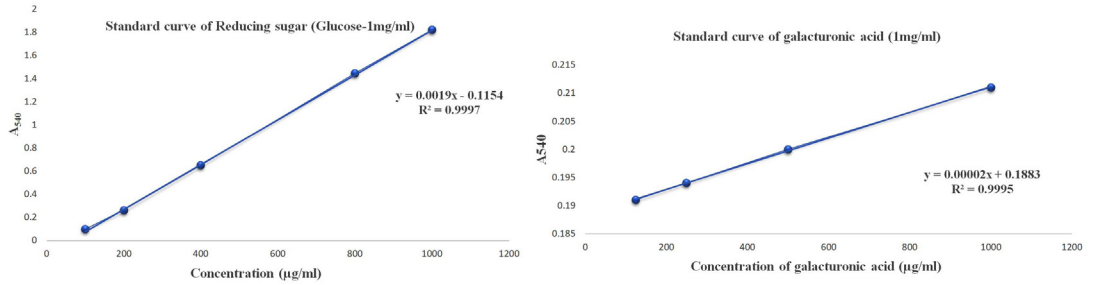

Figure 8. Standard curve of glucose and galacturonic acid

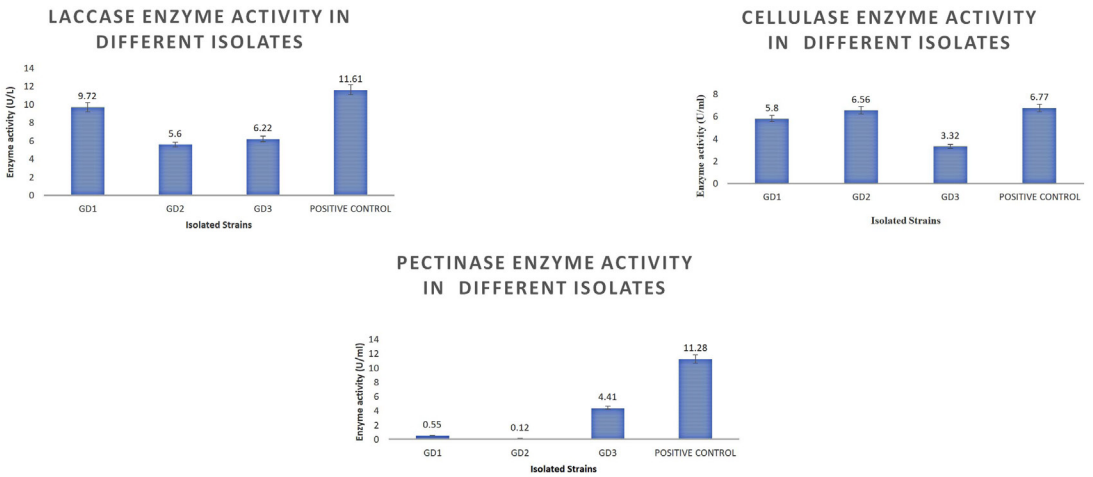

Figure 9. Comparison among GD1, GD2 and GD3 for laccase, cellulase and pectinase enzyme activity. Figure 9 depicts that GD1 shows the highest laccase activity, GD2 shows highest cellulase activity and GD3 shows the highest pectinase activity.

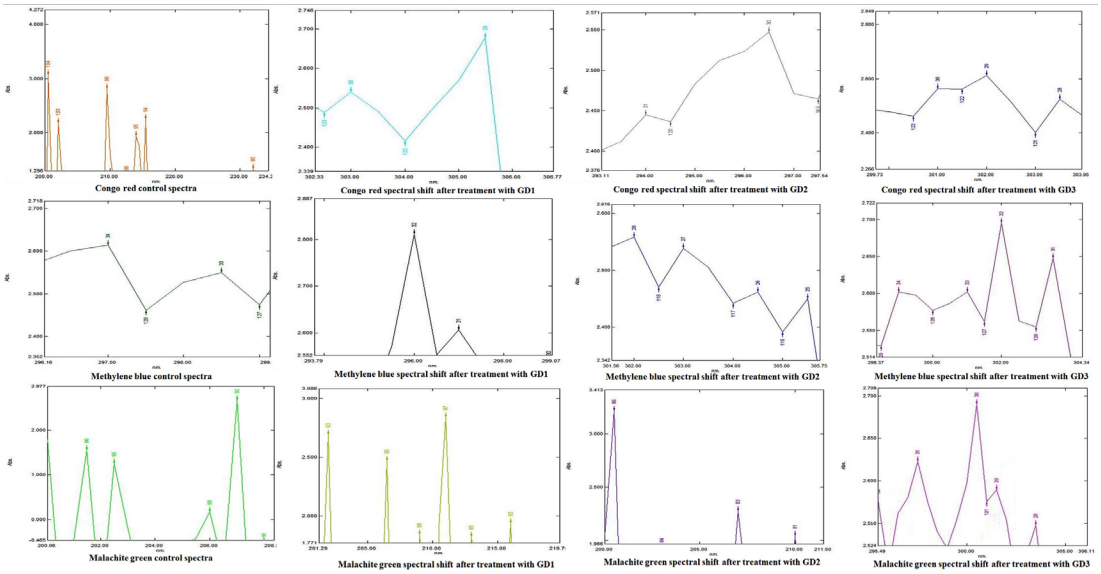

Figure 10. UV spectral analysis (before and after bacterial treatment with congo red, methylene blue, and malachite green). This figure depicts that before and after treatment of the dyes with different bacterial isolates GD1, GD2, and GD3, respectively, shows the change in the absorption spectral shift of the dyes that designate the breakdown of the dyes by the bacterial isolates (dye decolorization). Here Y-axis denotes absorbance and X-axis denotes wavelength in UV-range.

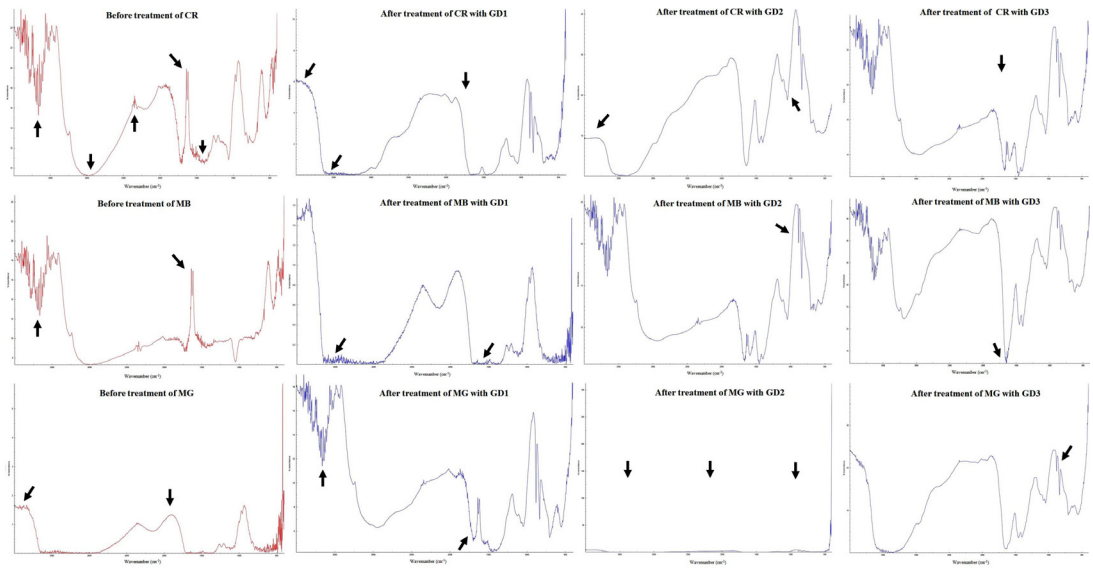

Figure 11. FT-IR spectral analysis of dyes congo red (CR), methylene blue (MB), and malachite green (MG) before and after bacterial treatment. This figure depicts that before and after treatment of the dyes with different bacterial isolates GD1, GD2, and GD3, respectively, shows the change in the transmittance spectra of the dyes that designate the breakdown of the dyes by the bacterial isolates (dye decolorization). Here Y-axis denotes %transmittance, and X-axis denotes wavenumber(cm^{-1}).[Range:4000-400 cm^{-1}].

new peaks had emerged while some entirely vanished. This might result from the degradation and transformation of the MB dye molecules by GD1, GD2, and GD3 bacterial isolates.[54]

For MG, particular peaks between 1500 and 500 cm^{-1} were associated with mono- and para-disubstituted benzene rings, supporting the C=C stretching phenomena of the benzene ring. The spectrum also exhibited peaks for the C-N stretching vibrations between 1100-1200 cm^{-1}. Indicating that the tertiary amines in MG underwent a transformation, the two bands initially situated between 2300 and 2400 cm^{-1} corresponded to the asymmetric and symmetric vibration of tertiary amine salt was disappeared after treatment by GD2. Further evidence for the complete elimination of aromatic rings for MG by GD2 came from the absence of peaks between 500- 3500 cm^{-1}. New breakdown product generation was also observed in the FTIR spectral change of treated MG by GD1 and GD3. Two bands between 3200 and 3500 cm^{-1} assigned to

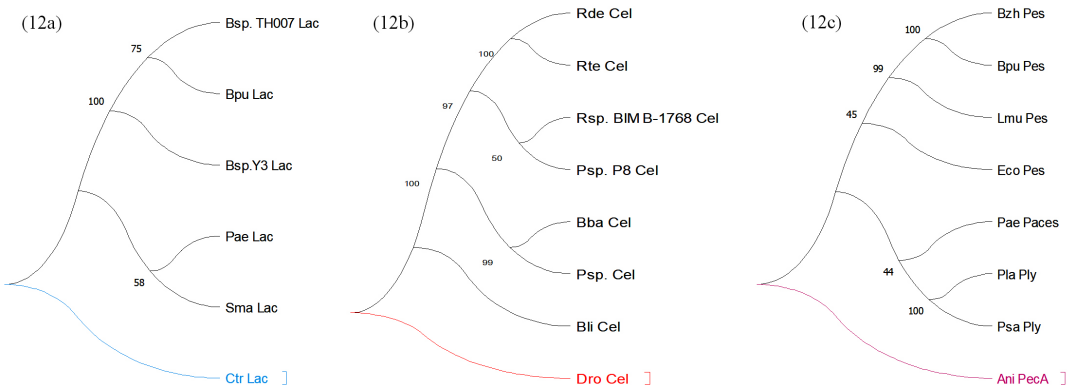

Figure 12. Phylogenetic trees of Laccase, Cellulase, and Pectinase producing most potential microbial strains considering bootstrap and outgroup applications. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates represents the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. Evolutionary analyses were conducted in MEGA11. [Lac: Laccase, Cel: Cellulase, Pes: Pectin esterase, Paces: Pectin acetyl esterase, Ply: Pectin lyase/Pectate lyase, PecA: Pectinase A]. (12a) This analysis involved 6 amino acid sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 710 positions in the final dataset. [Bsp TH007 : *Bacillus* sp. TH007 (>WP_058337369.1:1-287), Bpu: *Bacillus pumilus* (>WP_038475165.1:1-287), Bsp. Y3: *Bacillus* sp. Y3 (>WP_148780461.1:1-287), Pae: *Pseudomonas aeruginosa* (>OHQ50338.1), Sma: *Stenotrophomnas maltophilia* (>KUO99753.1), Ctr: *Colletotrichum trifolii* (>TDZ49563.1:1-697)]. (12b) This analysis involved 8 amino acid sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 641 positions in the final dataset. [Rde: *Roseateles depolymerans* (>WP_058936029.1:1-594), Rte: *Roseateles terrae* (>WP_088450018.1:1-594), Rsp. BIM B-1768: *Roseateles* sp. BIM B-1768 (>WP_261760547.1:11-596), Psp. P8: *Pelomonas* sp. P8 (>WP_233369739.1:1-595), Bba: *Burkholderiales bacterium* (>RTL39601.1:27-596), Psp.: *Pelomonas* sp. (>MBL8279287.1:1-547), Bli: *Bacillus licheniformis* (AOP15241.1), Dro: *Diplocarpon rosae* (PBP26369.1)]. (12c) This analysis involved 8 amino acid sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 520 positions in the final dataset. [Bzh: *Bacillus zhangzhouensis* (>WP_034319360.1:1-326), Bpu: *Bacillus pumilus* (>WP_127686996.1:1-326), Lmu: *Lachnospira multipara* (>SEF41635.1), Pae: *Pseudomonas aeruginosa* (>WP_079392931.1), Pla: *Pseudomonas lactucae* (>WP_205489322.1:1-380), Psa: *Pseudomonas salomonii* (>WP_069787611.1:1-380), Eco: *Escherichia coli* (>OEL43745.1), Ani: *Aspergillus niger* (QUS93879.1)].[Outgroups:Ctr: *Colletotrichum trifolii*, Dro: *Diplocarpon rosae*, Ani: *Aspergillus niger*].

N-H stretching vibrations of primary amines were seen in the FTIR spectra of dyes. These findings demonstrate the bacterial (GD1, GD2, and GD3) action in degrading dyes for MG.[55]

Analysis on phylogenetic state of microbial assemblages

A total of 5 sequences for laccase, 7 for cellulase, 7 for pectinase from all bacterial species, and 3 sequences from fungal species (supplementary dataset1) were selected for the current study depending on their laccase, cellulase, pectinase activity, ABTS-oxidation capacity for laccase and DNS assay capability (released reducing sugar) for cellulase and pectinase that showed similar enzymatic characteristics with the isolated strains[56,57](Table S1). After multiple sequence alignments by the MUSCLE algorithm, boot-strapping was done 1000 times in MEGA 11 software. Further Neighbour-Joining (NJ) method was used to design the phylogenetic tree. To check the reliability of an out-group sequence, *Colletotrichum trifolii, Diplocarpon rosae,* and *Aspergillus niger* were chosen for laccase, cellulase, and pectinase enzymes, respectively, depending on the homology similarity percentage. After that, clustering was performed to observe the out-group position in the clusters of sequences (Figure 12a,12b and 12c). Multiple sequence analysis provides multiple positions of conserved amino acid, which suggest that these conserved residues must have a significant function in the case of laccase, cellulase, and pectinase sequences and its evolution in bacteria. From the information on sequence alignment and the phylogenetic tree, it is confirmed that it can be used to design PCR (polymerase chain reaction) primers for laccase, cellulase, and pectinase gene isolation purposes. Further, it is necessary to add an out-group to root the phylogenetic tree, which must be an unrelated single or group of species that are not included in the group of species under the present study. Placing of out-group taxa was performed onto the phylogenetic tree somewhere below the ancestor of the whole taxa group. The bootstrap method was used to analyze the phylogenetic tree consistency, which is a statistical analysis procedure that re-samples the data and determines the strength of nodes on the tree. 100% bootstrap value indicates the most substantial support, and up to ≥ 90% signifies strong support. Values between 50% to 70% signify the accurate groupings that have been proved here.[40-41]

CONCLUSION

The current research effectively produced lignocellulolytic enzymes from the three most potential bacterial isolates, GD1, GD2, and GD3, among many other samples. They were able to produce laccase, cellulase, and pectinase in respective media along with the ability of dye decolorization in both plate assay as well as quantitative estimation assay from where we became able to understand the efficacy of each enzyme as they showed significant dye degradation within a very short period. Degradation of the dyes was proven by FTIR analysis, where the breakdown products were analyzed by their functional groups. Uv-spectral shift confirms decolorization due to breakdown effects by the potential enzymes of the isolated bacterial strains. The enzyme assay determined the presence and efficacy of all three isolates. Multiple sequence alignments and phylogenetic analysis of most potential bacterial groups for Laccase, Cellulase, and Pectinase showed the evolutionary relationship among each group of lignocellulolytic enzymes. Hence, this study concluded that all three isolated bacteria are potential candidates with multi-diverse applications, which can be applied to effectively treat solid and liquid waste in the dye, textile, pulp-paper, and fiber industries.

Future outlook

It is pretty clear from the literature review that laccase is a well-established dye-degrading enzyme. In the present study, cellulase and pectinase also showed the capacity for different dye degradation, like Congo red and malachite green. Here all the isolates cleared the media containing dye at a certain percentage that recommends using these enzymes soon for many other waste remediation purposes. We can use these enzymes in multiple industrial sectors like the jute industry, where jute fiber modification of fiber waste is highly demanding. We can also use these systems in the textile and pulp paper industry to reduce the organic load present in the effluents. Pectinase utilization is

highly appreciable as pectinase can lower the jute retting period. Pectinase can also be used in the fruit juice industry, where it is used for juice clearing. Very limited literature exists that depicts the function of pectinase in respect of dye decolorization. Hence this current study can prove the use of pectinase in waste remediation. As all three isolates can produce lignocellulolytic enzymes, they can act as a genetic consortium if we can use them in the proper place, along with proper nutrient supplements that can induce the enzyme activity. In the near future, the genetical characterization and the determination of enzyme kinetics, process parameter optimization of these respective isolates, and their modification by different wet and dry lab techniques will open the door to multi-diverse research opportunities to help to generate a pollution-free sustainable ecosystem.

SUPPLEMENTARY INFORMATION

Supplementary information accompanies this article at https://doi.org/10.22207/JPAM.17.1.59

Additional file: Additional Table S1.

ACKNOWLEDGMENTS

The authors would like to thank JIS University Kolkata and JIS Group Educational Initiatives. We are grateful to Empire Jute Mill Titagarh for providing financial support to carry out our work. We also express our sincere thanks to Dr. Goutam Pramanik and Dr. Aparna Datta, UGC-DAE Consortium for Scientific Research, Kolkata Centre, India, for obtaining FTIR data and discussion.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

Consultancy Research Project funded by Empire Jute Mill, Titagarh, West Bengal, India. Consultancy Research Project no NR. NO -015 Dated 01-10-18

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript and/or in the supplementary files.

ETHICS STATEMENT

Not applicable.

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