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ORIGINAL ARTICLE

Evaluation of potent marine ligninolytic bacteria and its efficiency in seawater-based delignification

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Abstract

The complex cross-linking between lignin and hemicellulose polymers in lignocellulosic biomass renders it recalcitrant and hinders an efficient conversion of fermentable sugars to bioethanol. In the present study, fourteen marine bacterial isolates collected from Digha, India (21.6222° N, 87.5060° E), were screened for their ability to utilize Kraft lignin in KL-MSM agar plates and the potent ligninolytic bacteria was selected and identified as *Shewanella chilikensis* LDB1 based on the 16S rRNA sequence analysis. The ligninolytic LDB1 strain was able to degrade 40.4% of Kraft lignin (1000 mg/L) in broth medium and also exhibited 91.66% decolorization of lignin mimicking dye, Azure blue (100 mg/L). Further, the LDB1 strain exhibited a maximum of lignin peroxidase and laccase activity of 18.67 U/ml and 4.83 U/L respectively. Further, seawater-based biodelignification of different lignocellulosic biomass using LDB1 strain resulted in reduction of lignin content from 29.15 to 20.28% in sugarcane bagasse, whereas in the case of rice straw and kans grass, the observed lignin removal was 23.70 to 16.42% and 25.33 to 22.58% respectively. The extent of delignification was further validated by enzymatic hydrolysis of the biopretreated substrates using commercial cellulase Cellic Ctec2 in seawater medium which resulted in a reducing sugar yield of 0.425 g/g, 0.498 g/g, and 0.314 g/g for rice straw, sugarcane bagasse, and kans grass respectively. The change in crystallinity and morphology of the biomass was monitored by X-ray diffraction study and scanning electron microscopy. These findings suggest that *Shewanella chilikensis* LDB1 could be potentially used in biodelignification and valorization of lignocellulosic biomass.

Keywords Seawater · Ligninolytic bacteria · Lignocellulosic biomass · Biodelignification · Saccharification

1 Introduction

The excessive use of fossil fuels has forced a shift from finite fossil-based energy to cleaner and inexhaustible energy as the prerequisite for spiraling global energy demand [1, 2]. The dearth of non-renewable fossil fuels has created the need of new energy sources and the sharp increase in biomass conversion to value-added products [3]. Therefore, the search for sustainable biomass that can serve as a source of low cost and eco-friendly energy production has gained prominence [4]. In this context, lignocellulosic biomass is deemed to be a potential candidate for the production of eco-friendly biofuel and value-added products [5].

Lignocellulosic biomass includes cheap, abundant, and year-around available raw material such as agricultural residues, plantation waste, forest waste, post harvesting, and organic waste [6]. Lignocellulose is mainly composed of carbohydrate polymers (cellulose and hemicellulose) which can be converted into fermentable sugar and a non-carbohydrate, polyphenolic aromatic polymer called lignin [6]. Lignin is a complex heteropolymer of phenyl propane units cross-linked via ether and carbon–carbon (C–C) bonds that engenders the recalcitrant property to plant cell wall and makes biomass unamenable for saccharification and fermentation [7]. In order to avoid this structural constraint, a suitable

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pretreatment is necessary to degrade the lignin. Over the decades, diverse physical, chemical, physicochemical, and biological pretreatment methods were employed for lignin alteration [8]. Physical, chemical, and physicochemical pretreatment are high capital investment method, employing harsh chemicals that causes sugar loss and also leads to the formation of inhibitors like furfural, hydroxymethyl furfural that hinders further saccharification, and fermentation processes [6, 9]. In addition, most of the pretreatment technologies such as steam explosion, microwave, and dilute acid–alkali method have not yet been adopted in industrial application for various drawbacks like special instrument requirement, high energy consumption, and acidic or alkaline waste water production [10, 11]. On the contrary, biological pretreatment is recently considered to be an preferred mode to enhance the conversion of lignocellulosic biomass to bioenergy, for its low chemical and energy use, and environmental friendliness [6]. In addition, utilization of microbes for delignification can subtly reduce the cost associated with chemicals and its related hazards to environment and human health [12]. Fungus and bacteria are well-known microbes that attack, depolymerize, and degrade lignin [13]. Fungi are more prevalent lignin degraders, such as white rot and few brown rot fungi; however, limiting growth conditions like long incubation period of 3–4 weeks and slower generation time limits their industrial implementation [12, 14]. Therefore, it becomes imperative to explore ligninolytic microbes outside the fungal kingdom. In this context, bacterial mediated degradation of lignin has not been explored to a great extent despite several bacteria such as *Pseudomonas* sp., *Streptomyces* sp., *Nocardia* sp., *Comamonas* sp., and *Bacillus* sp. were reported to be lignin degraders [15]. Further, bacteria harbor varied metabolic diversity, have short generation time and high environmental adaptability, and are known to tolerate wide range of pH, temperature, and oxygen. Also, the high surface-to-volume ratio of bacteria due to its microscopic size facilitates easy uptake of substrates in cell [12]. Among the various enzymatic activities associated with ligninolytic strains, laccase (Lac), lignin peroxidase (LiP), and manganese peroxidase (MnP) are the most widely investigated [16].

Further, the alarming scarcity of fresh water has shifted our focus to non-freshwater reserves such as seawater for biorefineries [4]. In spite of the abundant availability of seawater, the presence of salts in seawater severely impairs the efficiency of bacterial utilization for delignification. On the contrary, marine microbes are well-known to tolerate harsh industrial conditions [17]. But there is still a dearth of knowledge on the application of such marine microbes in biomass degradation.

Hence, the present study focused on the exploration of marine ligninolytic bacterial strains for biotreatment of lignocellulosic biomass for biorefinery purposes. Initially, different marine isolates were screened on the basis of their ability to metabolize Kraft lignin and the potent ligninolytic bacterial strain was chosen for further studies on decolorization and degradation of lignin mimicking dye Azure blue and Kraft lignin in broth medium. Based on 16S rRNA sequencing studies, the strain was identified and used for delignification of various lignocellulosic substrates in seawater medium. The change in lignin content upon biotreatment was estimated to determine the ligninolytic efficiency of the isolated strain. In addition, lignin peroxidase and laccase activity was carried out which is an intrinsic property of lignin degrading microorganisms. The efficacy of the LDB1 biotreatment strain was also examined by X-ray diffraction (XRD) and scanning electron microscopy (SEM) analysis of the untreated and biotreated substrates to understand the pretreatment induced structural and microscopic changes in biomass. Also, in order to validate the effect of biotreatment, cellulose release and reducing sugar yield was estimated by the saccharification biotreated substrates in seawater medium. Therefore, the findings of the present study elucidate the isolation of a novel marine ligninolytic bacterial strain for biodelignification of lignocellulosic substrates which could be a potential candidate for large-scale bioprocessing.

2 Materials and methods

2.1 Reagents

All chemical reagents and media components used in this study were of analytical grade and were purchased from Hi-media (Mumbai, India) and Sigma-Aldrich (USA). Kraft lignin (KL) was purchased from Sigma-Aldrich.

2.2 Isolation and screening of lignin-degrading (ligninolytic) bacteria

Seawater collected from Digha (21.6222° N, 87.5060° E), coastal area along the Bay of Bengal, India, was used for the isolation of marine microbes. Marine ligninolytic bacteria were screened on the basis of their growth at different concentrations of lignin on agar media. Briefly, 0.1 ml of the overnight grown bacteria (OD_{600nm} 0.1–0.2) was spread uniformly in Mineral Salt Medium (MSM) agar plates containing Na_2HPO_4 (2.4 g/L), K_2HPO_4 (2 g/l), NH_4NO_3 (0.1 g/L), $MgSO_4$ (0.01 g/L), and $CaCl_2$ (0.01 g/L), adjusted to pH 7.2 and supplemented with different concentrations of Kraft lignin (KL) (100 mg/L, 250 mg/L, and 500 mg/L). The plates were incubated for 14 days at 32 ± 2 °C.

The potent ligninolytic bacterial strain was selected based on the ability to utilize higher concentration of KL as manifested by the number of colonies in KL containing medium.

2.3 Phenotypic and biochemical characterization

Gram staining of the isolate was carried out according to the classical gram procedure [18]. The biochemical characterization studies were performed based on Bergey's Manual of Systematic Bacteriology [19].

SEM was used for studying the surface morphology of the selected strain as follows: 10 ml of LB broth culture of each of the isolate was centrifuged at 6000 rpm for 10 min at 4 °C and washed thrice with 0.1 M phosphate-buffered saline (PBS). Washed cells were fixed overnight at 4 °C with 2.5% glutaraldehyde prepared in 0.1 M PBS. Fixed cells were subsequently washed with PBS and dehydrated with increasing concentration of ethanol (30%, 50%, 70%, and 100%). The dehydrated samples were completely air-dried and examined under scanning electron microscope (JEOL JSM- 6480 LV).

2.4 Identification and phylogenetic analysis of the isolated strain

The potent strain was sequenced by Sanger method which was carried out by BioKart India Pvt. Ltd., Bangalore (India). The 16S rRNA sequence obtained was also submitted in GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) and the phylogenetic tree was made using MEGA X software version 11.0. The isolate was maintained on Zobella Marine agar (Himedia, India) and stored at 4 °C.

2.5 Growth study and salt tolerance of the isolated strain

The halotolerance of the strain was performed by estimating the growth in LB media containing different concentrations of salt (NaCl) ranging from 3.5 to 17%. Aliquots from the growth medium were taken regularly at an interval of 10 h and absorbance was recorded at 600 nm.

2.6 Decolorization of lignin mimicking dye—Azure blue by ligninolytic bacteria

The decolorization experiment was performed by inoculating 1 ml of overnight grown culture ($OD_{600nm} = 0.5$) in 100 ml of MSM media containing $(NH_4)_2HPO_4$ (1 g/L), KCl (0.2 g/L), $MgSO_4 \cdot 7H_2O$ (0.2 g/L), yeast extract (2 g/L), and glucose (2 g/L) with 100 mg/L of dye and incubated at 37 °C for over a period of 72 h. Samples were withdrawn at certain time intervals and centrifuged at 8000 rpm, 4 °C, 15 min for the separation of cell biomass. The bacterial pellet was resuspended into same amount of distilled water and the absorbance of cell suspension was measured at 600 nm. The Azure blue (AB) decolorization efficiency was measured at 650 nm [20]. Uninoculated flasks served as the control. The decolorization percentage is calculated as per Eq. 1.

$$Decolorization\ (\%) = \frac{Initial\ absorbance - Final\ absorbance}{Initial\ absorbance} \times 100 \quad (1)$$

LiP activity of the crude supernatant was measured by H_2O_2-dependent oxidation of veratryl (3,4-dimethoxybenzyl) alcohol to veratraldehyde at 310 nm [21]. The reaction mixture contained 0.1 ml crude enzyme, 0.5 ml of 100 mM sodium tartrate buffer (pH 3.8), 0.5 ml of 4 mM veratryl alcohol, and 0.1 ml of H_2O_2 and incubated at 30 °C for 5 min. The change in absorbance was recorded at 310 nm ($\varepsilon = 9300$ M/cm) [22]. One unit of LiP activity is defined as the amount of enzyme required to oxidize 1 µmol of veratryl alcohol to veratraldehyde per milliliter per minute. The enzyme unit is expressed as IU/ml.

2.7 Biodegradation and surface alterations of Kraft lignin by ligninolytic bacteria

For studying degradation and decolorization of KL, 1000 mg/L KL was added in MSM broth media, pH 7. Ten milliliters of overnight grown cells (OD 600nm = 0.5) was harvested by centrifugation, washed three times with sterile water, and inoculated in KL-MSM media. Uninoculated flasks containing same concentration of KL served as control. The flasks were incubated at 32 ± 2 °C, 150 rpm for 7 days, and samples were collected at an interval of 24 h for evaluating KL degradation, decolorization, and laccase activity.

Laccase activity of the crude supernatant was measured based on the oxidation of 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) to ABTS radical at 420 nm. The reaction mixture contained 0.3 ml of crude supernatant, 0.6 ml of 0.1M citrate buffer (pH 5), 0.3 ml of 5 mM ABTS, and 1.4 ml of distilled water. The change in absorbance was measured after 2 min incubation at 420 nm [23]. One unit of laccase activity is defined as the amount of enzyme required to catalyze 1 µmol of ABTS per minute. The enzyme unit is expressed as IU/ml.

After 7 days of incubation, control and biotreated KL sample were centrifuged at 12,000 rpm, 10 min. The cell free supernatant was vacuum dried to powder form and was used for studying morphological changes by scanning electron microscope (JEOL JSM-6480 LV). Further, Fourier transform infrared spectroscopy analysis (FTIR) analysis of KL was also performed for

Table 1 Screening of ligninolytic bacterial strains on MSM-KL media containing different concentrations of KL (100 mg/L, 250 mg/L, and 500 mg/L) as the sole carbon source

KL + MSM Agar media	Isolates from seawater			
	LDB1	LDB6	LDB9	LDB11
100 mg/L	+ + +	+ + +	+	+ +
250 mg/L	+ + +	+ +	−	+
500 mg/L	+ + +	+	−	−

+ = 50 colonies; + + = 50–75 colonies; + + + = more than 100 colonies; − = absence of colony

LDB lignin-degrading bacteria

studying the change in chemical bonds within the spectra range of 4000 to 400 cm^{-1} using Fourier transform infrared spectrometer (Thermofisher, Nikolet iS-10) at a resolution of 1.0 cm^{-1}.

2.8 Optimization of substrate loading for ligninolytic bacteria mediated delignification of lignocellulosic biomasses in seawater medium

Varying biomass loading (1.5%, 2.5%, 3.5%, and 4.5%) of three different substrates (rice straw, sugarcane bagasse, and kans grass) were used for biodelignification using ligninolytic bacteria in seawater reaction medium containing 1% yeast extract and 0.3% peptone. The seawater with a salinity of 35ppt was filtered and autoclaved at 121 °C for 15 min and was used for biodelignification studies. The substrates were inoculated with 10% (v/v) of overnight grown ligninolytic bacterial strain and were incubated at 32 ± 2 °C under constant agitation at 150 rpm for 7 days. Subsequently, the biotreated substrates were washed with distilled water, dried, and used for further experiments. Lignin and cellulose content of the untreated and biotreated biomass were obtained following the acetyl bromide method [24] and semi-micro determination method [25] respectively. Hemicellulose content of the substrates was measured following the colorimetric assay of hexoses and pentoses [26].

2.9 Characterization study of the untreated and biotreated substrates

2.9.1 X-ray diffraction

Crystallinity of the untreated and biotreated substrates were analyzed over a range of 5–50° (2θ) with a step size of 0.03° and scanning rate of 5°/min using Bruker AXS D8 Advance with Davinci Design. The crystallinity index (CrI) of the samples was calculated as per Eq. 2.

$$Cr1(\%) = \frac{\left(I_{002} - I_{am}\right)}{I_{002}} \times 100 \quad (2)$$

where CrI is the crystallinity index, I_{002} corresponds to the maximum intensity at crystalline region; I_{am} corresponds to the minimum intensity at amorphous region.

2.9.2 Scanning electron microscopy

Surface morphology of the untreated and biotreated samples were observed using scanning electron microscope (JEOL JSM-6480 LV) by mounting dried samples on metallic stub with adhesive coated with silver.

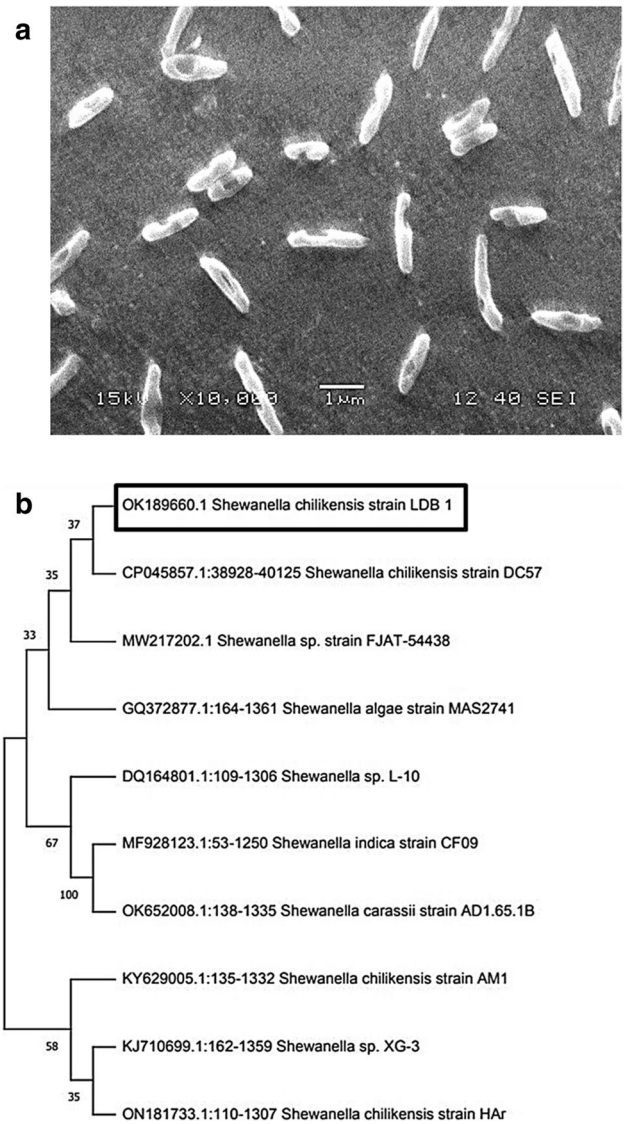

Fig. 1 **a** Scanning electron microscopy image of LDB1. **b** Phylogenetic tree of *Shewanella chilikensis* LDB1 associated with other members of the genus *Shewanella* using 16S rRNA sequence

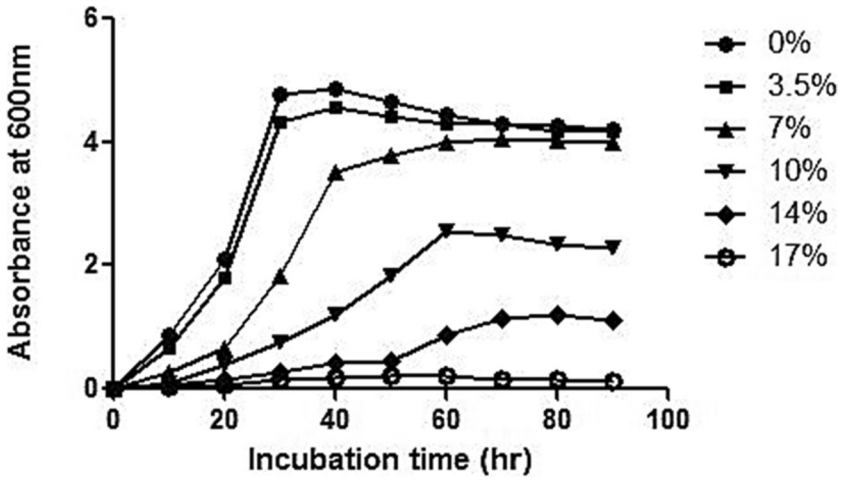

Fig. 2 LDB1 growth curve with different NaCl concentrations

3 Results and discussion

3.1 Isolation and screening of Kraft lignin–degrading bacteria

KL is a well-known lignin model compound and hence was used for biodelignification studies. Fourteen bacterial isolates from seawater were screened for their ability to utilize KL on KL-MSM agar plates supplemented with different concentration of KL (100 mg/L, 250 mg/L, and 500 mg/L). Four of the bacterial isolates (LDB1, 6, 9, 11) were found to utilize KL which was evident from the appearance of the colonies as shown in Table 1. However, at higher concentration of 500 mg/L, only LDB 1 was seen to have significant number of colonies and hence, it was selected for further studies.

3.2 Taxonomic characterization and identification of the isolate

The marine strain LDB1 was found to be gram negative, facultative anaerobe, motile bacteria with positive

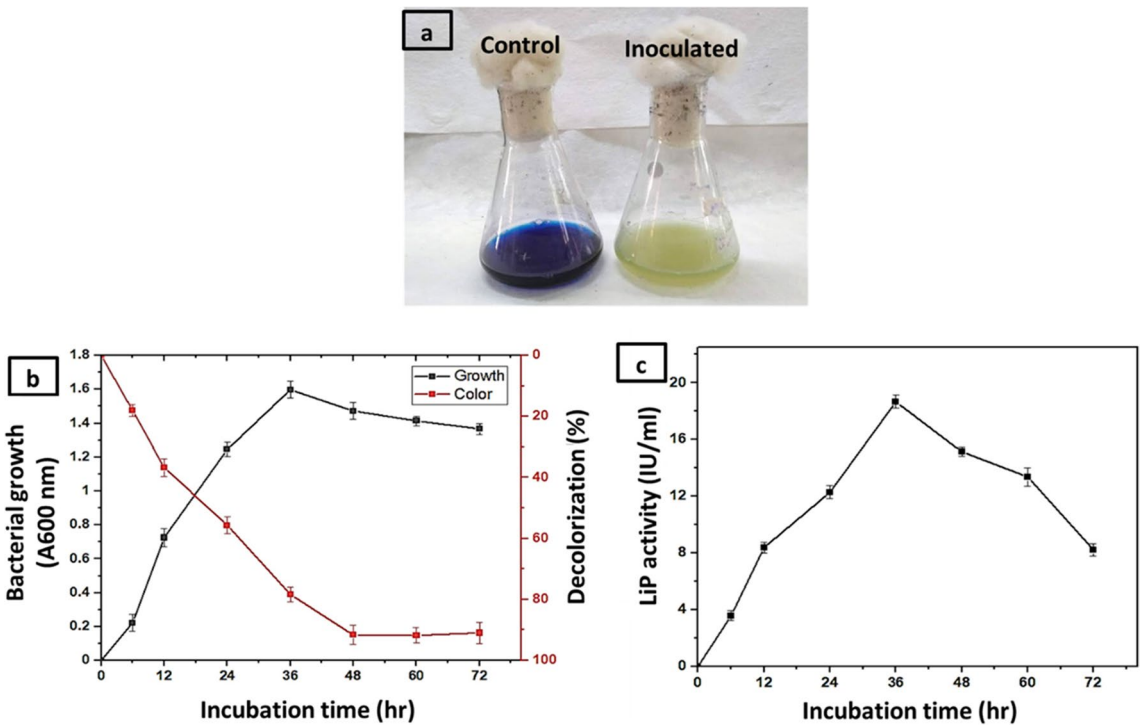

Fig. 3 **a** Decolorization of Azure blue dye (100 mg/L) by *Shewanella chilikensis* LDB1, **b** time course bacterial growth and dye decolorization, **c** and lip activity by LDB1

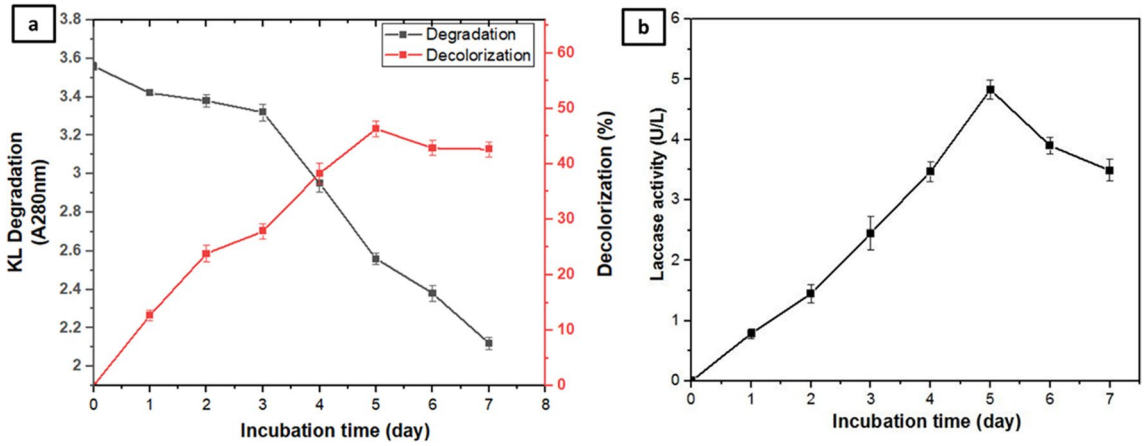

Fig. 4 **a** Time course of Kraft lignin (KL) degradation, decolorization and **b** laccase activity by LDB1

catalase and oxidase activity. SEM analysis of the strain revealed rod-shaped morphology (Fig. 1a). Taxonomic identification of the selected strain was carried out using 16S rRNA gene sequencing and BLAST analysis was performed to construct the phylogenetic tree using Neighbor-Joining method (Fig. 1b). Based on the sequence analysis and phylogenetic studies, the selected bacterium was identified as *Shewanella chilikensis* and designated as *S. chilikensis* LDB1. The 16S rRNA gene sequence was deposited in NCBI gene bank with accession no. OK189660. Recently, a marine bacteria *Shewanella baltica* JD0705 isolated from mangrove was found to exhibit potential ligninolytic ability and was utilized for biological delignification of rice husk powder [27].

3.3 Growth curve of isolated marine lignolytic bacteria

In order to examine the salt tolerance behavior of the marine ligninolytic strain *Shewanella chilikensis* LDB1, it was grown under different concentrations of NaCl (3.5–17%) (Fig. 2). The selected isolate was found to have optimum growth at 3.5% salt concentration with an extended exponential phase of 30 h following which the strain entered stationary phase. On the contrary, at higher salt concentrations (7–10%), delayed exponential phase was noticed along with longer stationary phase. At further higher salt concentrations (14–17%), a significant growth inhibition was observed. These results confirm the ability of bacteria to sustain and grow under varying salt concentration indicating its halotolerant character.

3.4 Biodegradation of lignin mimicking dye—Azure blue

In order to examine the biodegradation ability of the isolated *Shewanella chilikensis* LDB1strain, dye decolorization experiments were performed using Azure blue, which is a recalcitrant phenothiazinium azo dye and its decolorization is associated with the presence of LiP [28]. Experiments were conducted by inoculating the strain in MSM broth media containing Azure blue (100 mg/ml) and the bacterial growth and dye decolorization was monitored at 600 nm and 650 nm respectively for 72 h. The decolorization of Azure blue dye in broth medium was observed with no dye decolorization detected in control

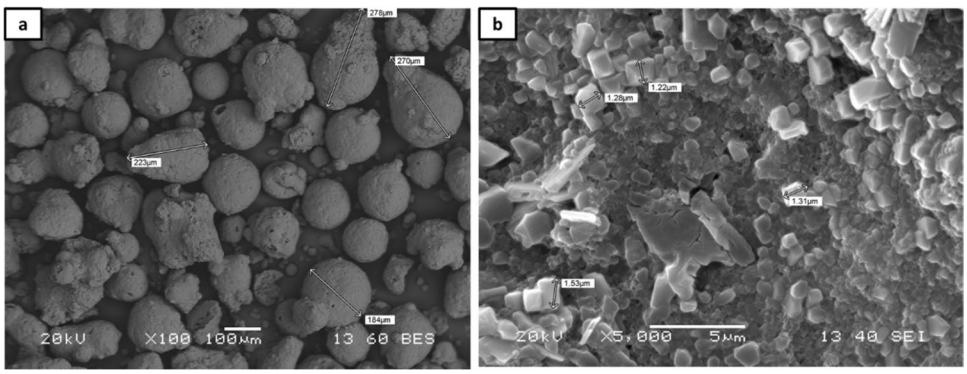

Fig. 5 Scanning electron microscopy (SEM) showing changes in Kraft lignin (KL); **a** untreated (×100) and **b** biotreated by LDB1 (×5000)

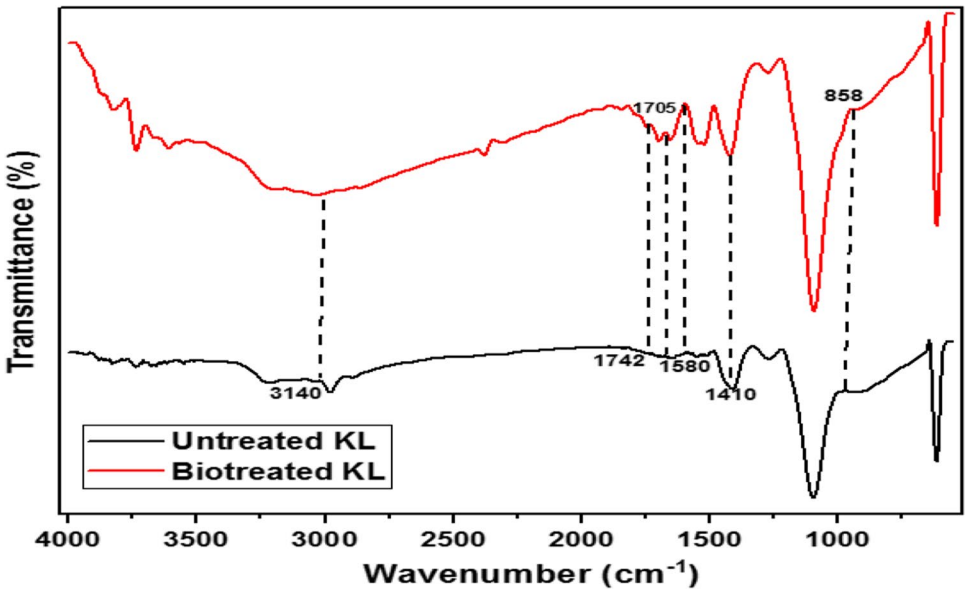

Fig. 6 FTIR spectra of untreated and biotreated Kraft lignin (KL)

(uninoculated) flasks (Fig. 3a). The results indicated that the strain exhibited maximal growth at 36 h during which dye decolorization was also found to be 78.33% and subsequently a decline in growth and dye removal was observed (Fig. 3b). Also, decolorization continued gradually at further time points and a total decolorization of 91.66% was observed after incubation of 48 h.

In order to validate these findings, LiP activity of the strain was also measured which was found to peak at 18.67 U/ml after 36 h (Fig. 3c). Therefore, these results confirmed that the isolated *Shewanella chilikensis* LDB1 strain harbors a plausible mechanism of lignin degradation. Such findings of Azure blue decolorization have been few and far between recent study due to the toxicity and recalcitrant nature of the dye [29]. The recalcitrant heterocyclic nature of Azure blue renders it resistant to oxidation by non-peroxidases, alcohol oxidases, manganese peroxidases (MnPs), or laccases (Lac) alone. Hence, its breakdown requires high redox potential agents like LiPs [20, 28]. However, previous reports of 93.5% and 96% Azure blue decolorization by *Bacillus* sp. MZS10 and *Serratia liquefaciens* respectively reaffirmed the relevance of the current study [29, 30].

3.5 Degradation and decolorization of Kraft lignin in broth media

Since the strain LDB1 was found to exhibit LiP activity, it was of interest to investigate the degradation of KL (1000 mg/L) in broth medium over a time period of 7 days. The results revealed that the bacterial strain was able to degrade 40.4% of KL after 7 days of incubation which was obtained from the decrease in absorbance at 280 nm (Fig. 4a). Further, bacteria-mediated decolorization of KL was also observed which was evident from the decrease in absorbance by 46.33% at 465 nm. The observed decolorization of the media may be attributed to the ligninolytic enzyme mediated depolymerization of lignin [31]. Earlier studies reported about 37% of KL degradation by bacterial species of *Novosphingobium* sp. B-7 [31]. Also, it was found that 41.5% of KL was removed after 7 days of pretreatment by *Cupriavidus basilensis* B-8 [32]. *Comamonas sp*. B-9 bacteria showed up to 47.3% KL depolymerization after 7 days of pretreatment [33]. Further, laccase activity was carried out at various time points (0–7 days) in the presence of 5 mM ABTS and the change in absorbance was measured at 420 nm. A gradual increase in absorbance was observed and the maximum laccase activity of 4.83 U/L was recorded on the 5th day (Fig. 4b). Therefore, these results establish the presence of ligninolytic activity in the isolated bacterial strain which facilitated the metabolism of KL in broth media.

3.6 Scanning electron microscopy and Fourier transform infrared spectroscopy analysis of untreated and biotreated Kraft lignin

The lignin depolymerization by the isolate was further confirmed by investigating the morphological changes by SEM. The untreated KL exhibited smooth surface with porous internal structure and diameter in the range of 184–278 µm as shown in Fig. 5 a. On the other hand, the morphology of the KL after 7 day biotreatment was found to be deformed and disintegrated with diameter reduced to 1.22–1.53 µm (Fig. 5b). Therefore, the change in shape and size of the biopretreated lignin suggests the significant breakdown of lignin by bacteria into smaller particles and confirmed the pretreatment-induced surface alterations. Also, similar structural changes have been previously reported with regard to the treatment

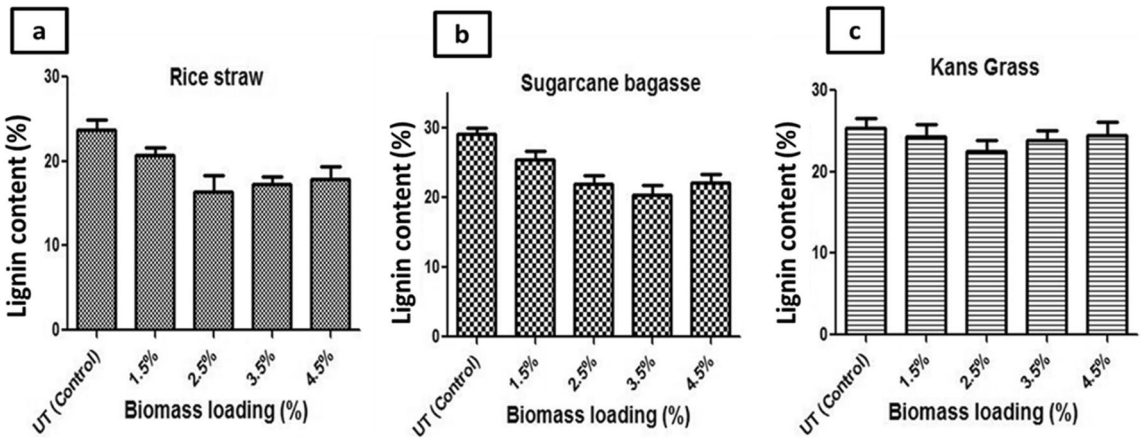

Fig. 7 Change in lignin content at different substrate loadings of lignocellulosic biomass; **a** rice straw, **b** sugarcane bagasse, and **c** kans grass by LDB1

of lignin by *Pandoraea* ISTKB, *Cupriavidus basilensis* B-8, *Bacillus ligniniphilus* L1, and *Comamonas* sp. B-9 [14, 32–34]. In a similar study, the degradation of KL by *Cupriavidus basilensis* B-8 after 7 days resulted in size reduction up to 10 µm [32]. The alterations in microstructure and increase in surface area caused by *Ochrobactrum oryzae* BMP03 were found to facilitate the subsequent hydrolysis by allowing the microbes and enzymes to penetrate easily thereby emphasizing the consequences of the pretreatment induced surface alterations in enzymatic saccharification [35].

Further, FTIR analysis of untreated and biotreated KL was carried out to investigate the changes in the functional groups upon biotreatment. A thorough examination of the FTIR spectra (Fig. 6) revealed that some of the bands present in untreated KL were lost upon pretreatment along with appearance of few additional bands in the FTIR spectra of pretreated KL. Previous insights on FTIR spectral analysis suggest that the band observed around 3500–3000 cm^{-1} could be due to the –OH stretching vibration of phenol, alcohols, and organic acids present in lignin [36]. Further, the spectra of biotreated KL showed prominent bands at region between 1800 and 500 cm^{-1} [34]. The transmittance observed at 1580 cm^{-1} indicates the aromatic skeleton vibration coupled with C = O stretching [37]. Also the band at 1410 cm^{-1} corresponds to the deformation of the C–H bonds and the band at 858 cm^{-1} represents the C–H vibrations imposed by guaiacyl units [34]. Further, the appearance of new bands at 1742 and 1705 cm^{-1} in biotreated KL suggests the degradation of KL during pretreatment [34]. Interestingly, a new band was also observed at 3140 cm^{-1} in pretreated KL. These results demonstrated the pretreatment-induced alterations in FTIR spectra which were consistent with the changes in morphological characteristics of KL indicated in SEM studies.

3.7 Optimization of substrate loading for LDB1 mediated delignification of lignocellulosic biomass in seawater medium

The lignin degradability of the ligninolytic strain LDB1 was further utilized for delignification of lignocellulosic substrates such as rice straw, sugarcane bagasse, and kans grass for a duration of 7 days. Initially, the delignification

Table 2 Delignification efficiency of various ligninolytic bacterial strains using different substrates

Substrate	Source (ligninolytic bacteria/enzyme used)	Time of incubation (day/s)	Lignin removal (%)	Reference
Rice straw	*Shewanellachilikensis*LDB1	7	30.71	This study
Sugarcane bagasse			30.42	
Kans grass			10.85	
Wheat straw	*Ochrobactrumoryzae*BMP03	16	44.47	Tsegaye et al. [15]
Rice straw		14	53.74	Tsegaye et al. [38]
Rice straw	Laccase from *Bacillus ligniniphilus*L1	1	8.93	Nazar et al. [40]
Tobacco straw	*Bacillus amyloliquefaciens*SL-7	15	28.55	Mei et al. [41]

Table 3 Compositional analysis and reducing sugar yield of untreated and biotreated substrates at respective biomass loading that showed maximum lignin removal upon biotreatment by LDB1

Substrates	Rice straw		Sugarcane bagasse		Kans grass	
	Untreated	Biotreated	Untreated	Biotreated	Untreated	Biotreated
Cellulose (%)	38.17 ± 1.34	46.77 ± 1.22	36.80 ± 1.47	50.44 ± 1.36	36.58 ± 1.45	44.34 ± 1.52
Hemicellulose (%)	20.36 ± 0.87	24.15 ± 1.34	26.43 ± 1.47	31.08 ± 1.36	24.13 ± 1.66	26.32 ± 1.52
Lignin (%)	23.70 ± 1.21	16.42 ± 1.93	29.15 ± 1.32	20.28 ± 1.38	25.33 ± 1.28	22.58 ± 1.36
Reducing sugar yield (g/g)	0.185 ± 0.02	0.425 ± 0.03	0.233 ± 0.03	0.498 ± 0.02	0.145 ± 0.01	0.314 ± 0.03

efficiency was evaluated by varying biomass loading (1.5%, 2.5%, 3.5%, and 4.5%) for each of the respective substrates and the optimal loading required for maximum delignification was determined. As expected, increasing the biomass concentration resulted in a gradual decline of lignin content in biotreated substrates with respect to untreated biomass (control) (Fig. 7). However, increasing the biomass loading by 4.5% hindered the delignification, probably due to overcrowding in the reaction medium. The biomass loading that resulted in maximum delignification were 2.5% for rice straw, 3.5% for sugarcane bagasse, and 2.5% for kans grass respectively. All these results conclusively established that the ligninolytic activity of the LDB1 strain could be utilized for delignification of lignocellulosic biomasses.

The lignin degrading potential of bacterial strains have been explored and tested with a wide range of substrates as shown in Table 2. Reports suggests that ligninolytic bacteria, *Ochrobactrum oryzae* BMP03 isolated from termite guts, exhibited lignin-degrading capability [38]. A substantial lignin degradation was reported upon biotreatment of rice straw using *Ochrobactrum oryzae* BMP03 for 14 days which resulted in a lignin removal of approximately 53.74%. Also, in a similar study, wheat straw was biodelignified by 44.47% by *Ochrobactrum oryzae* BMP03 when subjected to pretreatment

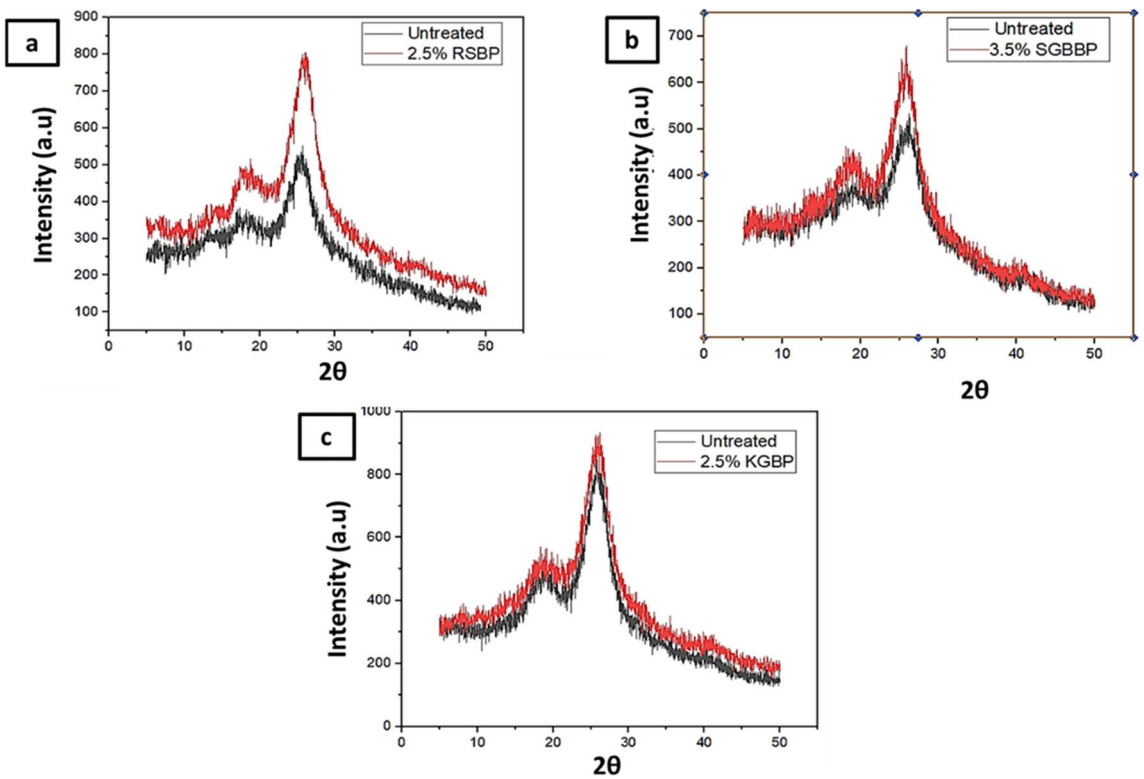

Fig. 8 X-ray diffraction (XRD) spectra of untreated and biotreated biomass; **a** rice straw, **b** sugarcane bagasse, **c** kans grass (RSBT, rice straw biotreated; SGBBT, sugarcane bagasse biotreated; and KGBT, kans grass biotreated)

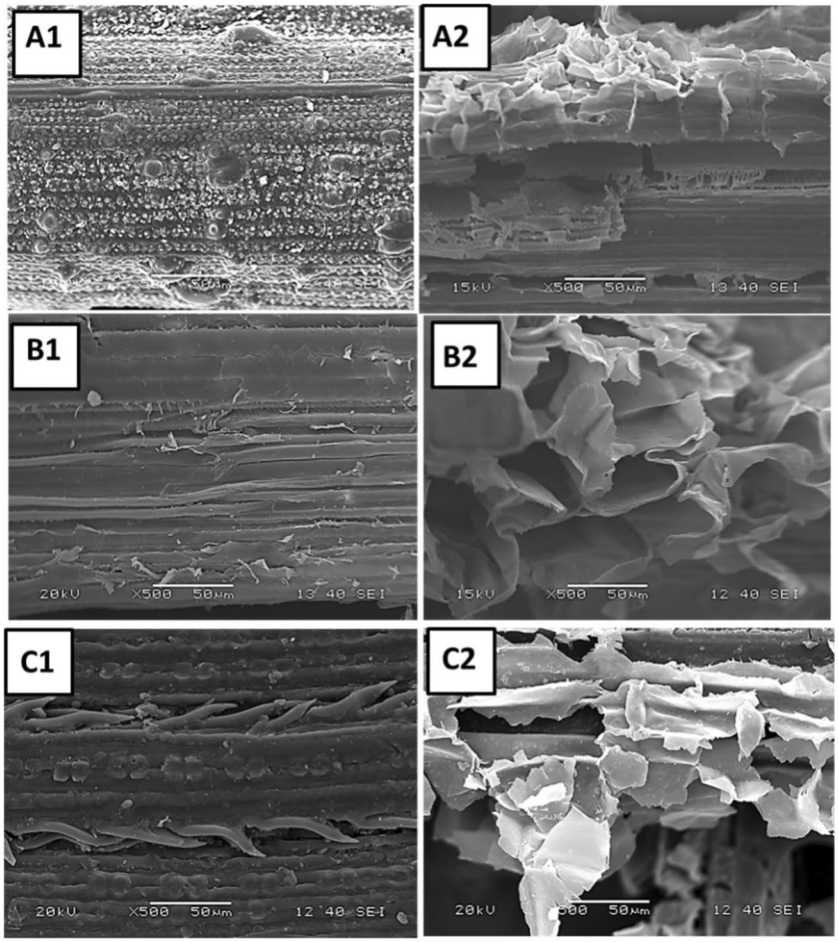

Fig. 9 SEM image of rice straw (A1, untreated; A2, biotreated), sugarcane bagasse (B1, untreated; B2, biotreated), and kans grass (C1, untreated; C2, biotreated)

for 16 days [15, 38]. Bacteria-mediated delignification studies from bamboo using *Echinodontium taxodii* 2538 strain after 30 days of pretreatment was shown to result in 29% lignin removal [39]. The ligninolytic potential of strain LDB1 corroborates with these findings and hence it needs to be explored further for the biorefinery applications.

3.8 Compositional analysis and reducing sugar yield of untreated and biotreated substrates

It is known that the removal of lignin barrier results in increased cellulose release [42]. Therefore, in order to determine the change in cellulose and hemicellulose content of the biotreated substrates, the respective percent biomass loading that showed maximum lignin removal (2.5% for rice straw and kans grass and 3.5% for sugarcane bagasse) was chosen for compositional analysis. As expected, the results revealed a substantial increase in cellulose content for all the substrates (Table 3). Interestingly, a marginal enhancement of hemicellulose was also detected. This suggested that the strain was able to effectively break down the lignin barrier and release the cellulose and hemicellulose content of the biomass. In a similar study, biodelignification of lignocellulosic biomass such as rice straw and wheat straw by *Ochrobactrum oryzae* BMP03 resulted in cellulose increase by 36.12% and 22.32% on 14 and 16 days of pretreatment. Further, it was found that nearly 394 mg of glucose and 113 mg of xylose/g of biopretreated corn stover were achieved by enzymatic saccharification using white rot fungus [43]. Thus, the LDB1-mediated release of cellulose and hemicellulose upon pretreatment in seawater medium validated its efficacy as a mode of delignification and can be supplemented with the more traditional acid or alkali-based pretreatment methods.

The extent of delignification and subsequent release of cellulose content was further tested by subjecting the biotreated substrates to enzymatic hydrolysis in seawater medium using commercial cellulase Cellic Ctec2. Untreated biomass (Control) was also hydrolyzed and the reducing sugar yield was used for comparison with biotreated substrates. As shown in Table 3, the reducing sugar yield obtained for biotreated rice straw, sugarcane bagasse, and kans grass was 0.425, 0.498, and 0.314 g/g respectively which was significantly higher

in comparison to their untreated forms. These results demonstrated that the delignification efficiency of *Shewanella chilikensis* strain in the current study facilitated the enzymatic hydrolysis and subsequent release of sugars.

3.9 Characterization studies of the biomass using XRD and SEM analysis

Crystallinity index is an important criterion that measures the relative availability of crystalline cellulose after pretreatment. Higher removal of lignin and amorphous polysaccharides can be deciphered from the higher value of crystallinity index. In the present study, the cellulose crystallinity of biotreated biomass was compared with untreated biomass employing XRD technique. XRD spectra of untreated and biotreated substrates are shown in Fig. 8. An apparent increase in cellulose crystallinity index was observed in all the biotreated substrates which can be attributed to the increase in crystalline cellulose along with concomitant decrease in lignin. Upon biotreatment, the crystallinity index was increased from 34.9 to 41.1%, 28.4 to 38.3%, and 38.5 to 43.3% for rice straw, sugarcane bagasse, and kans grass respectively. These results revealed that *Shewanella chilikensis* LDB1 could efficiently degrade lignin from lignocellulosic biomass in seawater medium. Also, the findings of the current study is consistent with bacterial-mediated pretreatment of cotton stalk by *Mycobacterium smegmatis* LZ-K2 [44] and rice straw and wheat straw pretreatment by *Ochrobactrum oryzae* BMP03 [15, 38].

Scanning electron microscopy was used to characterize the structural integrity and morphology of different lignocellulosic biomass after pretreatment with *Shewanella chilikensis* LDB1. Figure 9(A1, B1, C1) reveal the smooth, rigid, and highly arranged surface structure of untreated native biomass. On the contrary, surface of the biotreated biomass (Fig. 9(A2, B2, C2)) showed numerous alterations along with the separation of microfibrils implying the pretreatment-induced disruption of the lignocellulosic matrix. Interestingly, similar microstructural changes of the biomass were obtained when rice straw and wheat straw were exposed to *Ochrobactrum oryzae* BMP03 [15, 38].

4 Conclusion

Isolation and screening of potent ligninolytic isolates from seawater was carried out and most efficient strain was selected and identified as *Shewanella chilikensis* LDB1 based on 16 S rRNA sequencing. The bacterium was able to degrade a lignin mimicking dye, viz. Azure blue and found to be exhibit lignin peroxidase (Lip) activity of 18.67 U/ml. The strain was able to metabolize kraft lignin (KL) in broth medium and exhibited laccase activity of 4.83 U/L. In addition, the LDB1 strain was able to grow optimally in LB medium supplemented with 3.5% NaCl suggesting its halotolerant nature. These findings justified the use of LDB1 strain for seawater-mediated delignification of lignocellulosic substrates and subsequent release of cellulose and reducing sugars. The study further revealed that LDB1 strain was capable of efficiently degrading lignin from biomass by seawater-based biotreatment. A concomitant increase in cellulose release and reducing sugar yield of the biotreated substrates was observed and the resulting changes in crystallinity and microstructure were demonstrated by analytical techniques (XRD and SEM). The efficacy of the ligninolytic bacterial strain renders it a potential candidate for large-scale bioprocessing. However, additional experiments need to be carried out to further enhance and optimize lignin degradation.

Author contribution Conceptualization, B.D. and B.P.; methodology, B.D.; data analysis and investigation, B.D. and A.B.; resources, S.K. and B.P.; writing—original draft preparation, B.D. and A.B.; writing—reviewing and editing, B.P.; funding acquisition, S.K. and B.P.

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Data availability The data will be made available on request.

Declarations

Ethical approval Not applicable.

Competing interests The authors declare no competing interests.

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