Biodegradation of alkali lignin by *Bacillus flexus* RMWW II: analyzing performance for abatement of rice mill wastewater

Anuj Kumar, Rashmi Priyadarshinee, Subhajit Singha, Bratin Sengupta, Abhishek Roy, Dalia Dasgupta and Tamal Mandal

ABSTRACT

A potential bacterium *Bacillus flexus* RMWW II has been isolated from rice mill effluent, and examined for its decolorizing potential for lignin-mimicking dyes. The biodegradation of alkali lignin by the rod-shaped, Gram-positive, oxidase and catalase-positive *Bacillus flexus* RMWW II bacteria is due to its uptake of lignin as the sole carbon source. The lignin degradation was 100% at a lignin concentration of 50 mg L^{-1} but the degradation reduced to 20% at 400 mg L^{-1}. The bacterial-mediated biodegradation of alkali lignin was suitably explained by the Edward kinetics model with a maximal specific biodegradation rate (q_{max}) of 0.056 h^{-1} and true specific biodegradation rate (q^*) of 0.042 h^{-1}. The non-toxic nature of the metabolites of alkali lignin after bacterial degradation was illustrated by phytotoxicity studies. This bacterium was utilized to treat complex rice mill wastewater, as lignin is one of the major components of the effluent. A considerable reduction of 84% of chemical oxygen demand (COD) was observed in a batch reactor in 70 h of operation. The bacterial treatment results for the actual rice mill effluent indicate that *Bacillus flexus* RMWW II could be a promising agent for microbial remediation of lignin-laden raw rice mill wastewater.

Key words | alkali lignin, *Bacillus flexus*, biodegradation, kinetics, phytotoxicity, rice mill wastewater

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NOMENCLATURE

K_S, K_i	substrate-affinity constant and substrate-inhibition constant applied to the growth rate (mg L^{-1})
K'_S, K'_i	substrate-affinity constant and substrate-inhibition constant applied to specific degradation rate (mg L^{-1})
q	specific degradation rate $(g(g{\cdot}h)^{-1})$
q^*	apparent specific degradation rate $(g(g{\cdot}h)^{-1})$
q_{max}	maximum specific degradation rate $(g(g{\cdot}h)^{-1})$
S	AL concentration (mg L^{-1})
S_m	AL concentration at which $\mu = \mu_{max}$ (mg L^{-1})
S'_m	AL concentration at which $q = q_{max}$ (mg L^{-1})
X_0	initial biomass concentration (mg L^{-1})
X	bacterial biomass concentration (mg L^{-1})

GREEK SYMBOLS

μ	growth rate (h^{-1})
μ^*	fitting parameter, apparent maximum growth rate (h^{-1})
μ_{max}	true maximum growth rate (h^{-1})

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INTRODUCTION

Industrial developments are often penalized by the expenditure associated with the abatement of the contaminant load. Biotechnological methods are green technologies that ensure better aesthetics and a healthier environment while assisting the industries to minimize toxic compounds. Parboiled rice mills release a huge quantity of contaminated wastewater into natural aquatic bodies. Lignin and its derivatives are the principal pollutants in the rice mill effluent, which on discharge impart dark coloration and toxicity to the aquatic bodies (Kumar *et al.* 2016a, 2017a).

Alkali lignin (AL) is a toxic bio-refractory model organic pollutant that is more recalcitrant in structure than plant lignin (Chakar & Ragauskas 2004). Besides, the persistent nature of AL and its derivatives, which on accumulation turn the water unrecyclable, deteriorating product quality, also brings serious impacts on aquatic environments. Therefore, it is of the utmost urgency to degrade the AL. Lignin-utilizing organisms may act as an eco-friendly technology for the degradation of xenobiotic compounds into simpler components. Fungi belonging to white-rot or brown-rot families are the most studied microbes in lignin degradation (Sanchez 2009). Fungal species have the problem of choking the treatment plant's sludge line, they have a requirement for an exogenous carbon source and higher retention time for degradation of wastewater (Swamy & Ramsay 1999). These make fungal species unfavorable for large-scale use in a treatment plant. Bacteria can play an important role as a preferential resource of biodegradation owing to their ability to act on a broader range of physicochemical parameters (Bugg *et al.* 2011). In recent years, the ability of few bacterial strains such as *Azotobacter, Bacillus megatherium*, *Pseudomonas fluorescens, Planococcus* sp. and *Pandoraea* sp. has been reported in the biodegradation of industrial lignin (Minussi *et al.* 2007). However, inadequate research is present for the bacterially mediated abatement of AL as well as treatment of real wastewater from rice mills. The selection of a suitable strain having the ability to proliferate during the course of the bio-augmentation process may considerably reduce the expense and simplify the operating process.

The current research made an attempt to explore the detailed kinetic study (Haldane model, Aiba model, and Edward model) for the biodegradation of AL and treatment of rice mill wastewater by a bacterial strain isolated from rice mill effluent. The presumed ligninolytic activity of the newly isolated strain was illustrated by the decolorization of lignin mimicking dyes that bear structural resemblance to the variable and complicated lignin compound. AL is structurally different from actual plant-derived lignin, including modification of side chain and alkyl-alkyl cleavage, but still, it has been traditionally used in literature to understand the degradation of microbial strains (Morii *et al.* 1995). While many microbial strains can degrade lignin, not all of them can degrade AL, hence the degradation of AL is a good test for lignin-degrading capacity (Vicuna *et al.* 1993). Degradation studies to understand the kinetics of bacterial activity were carried out in synthetic AL solution, while actual rice mill wastewater was employed to understand the actual degradation behavior. Phytotoxicity assay was done to check the extent of toxicity of the metabolites of AL reduction. The overall objective was to isolate one bacterial strain that can be used for bacterial-remediated treatment of rice mill wastewater.

MATERIALS AND METHODS

Chemicals and reagents

A stock 10,000 mg L^{-1} of AL and mineral salt medium (MSM) was prepared in autoclaved distilled water (pH adjusted to 12). MSM consists of 0.25 g L^{-1} ammonium sulfate ($(NH_4)_2SO_4$), 0.42 g L^{-1} dipotassium hydrogen phosphate (K_2HPO_4), 0.42 g L^{-1} potassium dihydrogen phosphate (KH_2PO_4), 0.18 g L^{-1} magnesium chloride ($MgCl_2{\cdot}7H_2O$), 0.08 g L^{-1} calcium chloride ($CaCl_2{\cdot}2H_2O$) and 0.46 g L^{-1} ferrous sulfate ($FeSO_4{\cdot}5H_2O$), which was used for the growth of the microorganisms. The pH of the media was adjusted to 7.0 ± 0.2. Pure analytical grade AL of average molecular weight ~10,000 Da was used and brought from Sigma Aldrich. All other reagents were of analytical grade procured from Hi-Media and Merck, India, and were used without further purification. *Vigna radiata* used for the toxicity study has been procured from the local market of Durgapur, West Bengal, India.

Isolation and culture conditions

The lignin-degrading bacterium was isolated from rice mill wastewater samples obtained from the Lakshmi Shree rice mill, Alamganj, West Bengal, India. A 5 mL sample of rice mill wastewater was emptied into a 250 mL Erlenmeyer flask containing 100 mL of autoclaved MSM supplemented with AL (15 mg L^{-1}) as the carbon source. The flasks were incubated at 35 °C in a biological oxygen demand (BOD) incubator shaker at 140 RPM for 96 h. The turbid culture (5 mL) was then transferred to 100 mL of fresh MSM medium and incubated for the next 96 h. The whole process was performed four times. Subsequently, the enriched culture at 10^{-6} dilution was inoculated on nutrient agar media with 25 mg L^{-1} of lignin. Three morphologically distinct colonies were picked and re-streaked separately on fresh nutrient agar plates with a lignin concentration of 50 mg L^{-1}. The isolated colonies from the rice mill wastewater were designated as RMWW I, RMWW II and RMWW III, respectively.

Screening via decolorization of lignin model dyes in the batch liquid cultivation

The screening of efficient ligninolytic isolates can initially be detected by the decolorization of synthetic lignin model dyes that have structural similarities with lignin (Kiiskinen *et al.* 2004). Decolorization of the lignin-mimicking dyes, malachite green (MG), phenol red (PR), Azure B (AB), Toluidine blue (TB) and Congo red (CR) by three isolated strains was performed in 100 mL nutrient broth containing 5 mg L^{-1} dyes. Samples were withdrawn after 24 h of incubation and centrifuged for 15 min at 6,000 RPM at 4 °C. The decolorization of a definite dye was estimated as a percentage of the initial absorbance at λ_{max} (AB, 650 nm; PR, 435 nm; TB, 635 nm; MG, 615 nm; CR, 470 nm). Control experiments were performed in the same medium without bacteria. Color removal was calculated as decolorization (%),

$$\text{Decolorization (\%)} = \frac{(A-B)}{A} \times 100 \quad (1)$$

A is the absorbance of the initial dye solution and B is the absorbance at cultivation time, t.

Morphological and physiological characterization

Biochemical and physiological characteristics of the selected bacterial isolate were determined by IMViC tests and Gram staining using established protocols (Pushkar *et al.* 2019). Further identification using 16S rDNA sequencing was performed by Samved Biotech, Ahmedabad, India.

Biodegradation of alkali lignin

Prior to performing biodegradation, the pure culture was pre-grown overnight up to late exponential phase in nutrient broth at 35 °C and 140 RPM. The culture was centrifuged at 6,000 RPM for 15 min. The harvested pellet was washed and resuspended in PBS (phosphate-based saline) buffer (pH ~ 7.4). The 10× PBS buffer was prepared by dissolving 800 g NaCl, 20 g KCl, 144 g $Na_2HPO_4{\cdot}2H_2O$, 24 g KH_2PO_4 and 8 L of distilled water. The cell pellet was then inoculated into the MSM media with varying initial concentrations (25–400) mg L^{-1} of AL. At different interims, the residual lignin in the supernatant was assessed by acidifying with HCl, followed by subsequent extraction with ethyl acetate (Chandra *et al.* 2007). The sample that was extracted was dissolved in high-performance liquid chromatography (HPLC) grade acetonitrile, filtered using a 0.22 μm membrane filter (Millipore, India). The obtained filtrate was studied by HPLC (Waters™ 600, USA) furnished with a C18 reversed-phase column (250 × 4.6) mm and involving the mobile phase of acetonitrile/buffer (1:1 v/v) at a flow rate of 1 mL min^{-1}. A UV-visible detector analyzed the chromatogram at 280 nm.

Kinetics analysis

The kinetic study is essential for the scale-up of any reaction process (Kumar *et al.* 2017b). Kinetics of bacterial growth for AL degradation by the selected bacterial strain in the batch reactors may be described as:

$$\frac{dX}{dt} = \mu X - K_d X \quad (2)$$

K_d can be assumed to be negligible during exponential growth. Hence, Equation (2) can be written as:

$$\frac{dX}{dt} = \mu X \quad (3)$$

$$\mu = \frac{1}{X}\frac{dX}{dt} \quad (4)$$

The specific growth rate, μ (h^{-1}), is calculated from the linear plot of biomass (X) vs ln (S/S_0), where S_0 and S (mg L^{-1}) are the initial and the instantaneous concentrations of AL at time t. The important growth parameters such as maximum specific growth rate (μ_{max}), true maximum specific growth rate μ^* and S_m (mg L^{-1}) which is at the AL concentration at which $\mu = \mu_{max}$ were calculated using Equations (2)–(4), respectively (Christen *et al.* 2012).

The growth rate (μ) of microbial cells on inhibitory substrates in a batch reactor is mostly explained by Haldane's kinetic model as shown in Equation (5) (Christen *et al.* 2012).

$$\mu = \frac{\mu_{max} S\left[1 + \left(\frac{S}{K_s}\right)\right]}{S + K_s + \left(\frac{S^2}{K_i}\right)} \quad (5)$$

K_s (mg L^{-1}) and K_i (mg L^{-1}) are the affinity constant and the inhibition constant. The specific growth rate, μ (h^{-1}) is determined from the linear plot of log bacterial biomass against time and the slope of the curve was calculated using Equation (6).

$$\ln\left(\frac{X}{X_o}\right) = \mu t \quad (6)$$

The μ_{max} occurs when $d\mu/dS = 0$ at,

$$S_m = \sqrt{K_s K_i} \tag{7}$$

Similarly, the specific degradation rate 'q' was calculated from the gradient of a semi-logarithm plot of AL concentration, S vs. time (t) for each initial substrate concentration. Equations (5) and (6) were also employed to estimate the maximum specific degradation rate, q_{max} (h^{-1}), true maximum specific degradation rate q^* (h^{-1}), S'_m (mg L^{-1}, substrate concentration at $q = q_{max}$) substrate-affinity constant K'_s (mg L^{-1}).

Aiba model (Aiba *et al.* 1968)

$$\mu = \frac{\mu^* S}{K_s + S} exp\left(\frac{-S}{K_i}\right) \tag{8}$$

Edward model (Edwards 1970)

$$\mu = \mu^* S\left[exp\left(\frac{-S}{K_i}\right) - exp\left(\frac{-S}{K_s}\right)\right] \tag{9}$$

Kinetics models of Haldane, Aiba, and Edward were fitted to the experimental data obtained for bacterial growth and AL biodegradation. The values of μ^* and q^* are obtained from the line fitting and from these values μ_{max} and q_{max} are calculated using the following equation.

$$\mu_{max} = \frac{\mu^*}{1 + 2\frac{\sqrt{K_s}}{K_i}} \tag{10}$$

All the experiments were carried in triplicates and the results were presented as the mean value ± standard deviation to the purpose of accuracy. The sum of squared error (SSE) was considered as the indicator of goodness of fit of the model.

Rice mill wastewater treatment

Rice mill wastewater was collected from Laxmi Shree rice mill of Burdwan, West Bengal, India, followed by immediate storage of rice mill wastewater at 277 K. The obtained physicochemical values of rice mill wastewater were pH, 5.8; chemical oxygen demand (COD), 1,586 mg L^{-1}; color, yellowish; odour, obnoxious; lignin, 148 mg L^{-1}; phenol, 16 mg L^{-1}.

Acclimatized selected strain of *Bacillus flexus RMWW* culture (20 mL) was centrifuged at 10,000 RPM, 15 min to collect the microbial pellet. The pellet of bacterial cells was added in 100 ml working volume of wastewater in a 500 mL flask with 10% of MSM, pH 7 and subsequently incubated at 35 °C, 130 RPM in a shaker. At a regular intervals of time, the samples were centrifuged. The clear supernatant was examined for the determination of residual COD and other parameters of rice mill wastewater. The pellet was resuspended in phosphate buffer and utilized for the determination of bacterial growth at $OD_{600\,nm}$. Simultaneously, the control sets without bacterial culture were monitored to determine the abiotic effect. All the experiments were run in triplicates and results were expressed as the average of three experimental sets.

Analysis

The COD of samples was determined as per the standard protocol of APHA (Eaton *et al.* 2005) as well as reactor digestion procedure for a COD range of 0–1,500 mg L^{-1} employing an automatic COD analyzer (LoviBond, Germany). Phenol was measured using the colorimetric method of Folin–Ciocalteu (Ainsworth & Gillespie 2007) and color of the wastewater was examined, as explained by Bajpai & Bajpai (1994). The concentration of lignin was analyzed by Pearl and Benson's method (Pearl & Benson 1990).

Cell concentrations in the samples were analyzed by measuring the optical density (OD) at 600 nm using a UV–vis spectrophotometer (Agilent Technology, Cary 60) with the culture medium as a blank. $OD_{600\,nm}$ values were then converted to dry cell mass (mg L^{-1}) using a calibration curve between the dry cell mass (mg L^{-1}) and the optical density (600 nm).

Fourier transform infrared (FTIR) study

Changes in AL as a growth substrate and structural analysis of untreated, and bacterial-treated AL were determined using FTIR spectroscopy. For FTIR analysis, both control and bacterial broth containing lignin were taken out and diluted to 200 mg L^{-1}, and then centrifuged at 10,000 RPM for 30 min. The supernatant from both the control and the bacterial degraded lignin were taken in a glass Petri dish and dried overnight at 60 °C for 48 hours to evaporate all the water. The dried powder sample was then used for FTIR evaluation. All the dried samples were then analyzed by FTIR system using the KBr disc method, Bruker Tensor spectrophotometer in the 4,000–400 cm^{-1} wavenumber range.

Phytotoxicity assessment

A toxicity study of untreated and treated samples was performed by using a mung bean (*Vigna radiata)* germination bioassay. Ten healthy equal-sized seeds were surface-sterilized with 0.1% $HgCl_2$ and 70% ethanol followed by 2–3 time washing with distilled water. Aquaguard water treated seeds were considered as control. Seeds were arranged on filter paper placed in each petri dish which was then exposed to respective treated and untreated solutions. Plates were incubated at $25 \pm 3\,^{\circ}C$ and growth was observed for five days exposure with different concentrations of AL (6.25%, 12.5%, 25%, 50%, and 100%, where 100% lignin represents 250 mg L^{-1}) compared to control (Aquaguard™ water).

RESULTS AND DISCUSSION

Selection of potential isolate based on efficient decolorization of lignin model dyes

The isolated bacteria illustrated decolorization of a broad range of synthetic lignin-mimicking dyes, particularly phenothiazinium dyes such as AB, MG, and PR through liquid cultivation. Out of the three isolated bacteria, the strain *Bacillus flexus* RMWW II was selected, which depicted maximum dye decolorization as shown in the quantitative assay depicted in Figure 1. Interestingly, Azure Blue and Phenol reds are the well-known substrates for lignin peroxidase and manganese peroxidase, respectively (Arora & Gill 2005). The decolorization ability of the strain substantiated that the bacteria might inherit the catabolic activity for the delignification process (Rahmanpour *et al.* 2016). There was no appreciable change detected in the control experiments.

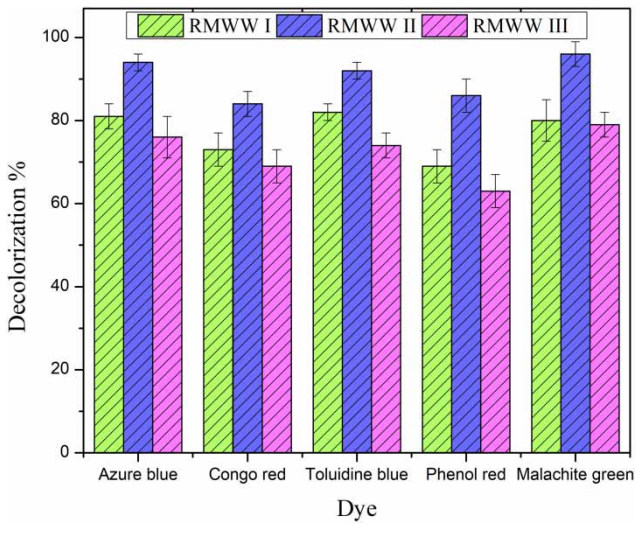

Figure 1 | Quantitative assay for demonstration of ligninolytic activity by selected strains – *Bacillus flexus* RMWW I, RMWW II and RMWW III on nutrient broth containing 5 mg L^{-1} of lignin mimicking dyes (Azure blue, Congo red, Toluidine blue, Phenol red, Malachite green) within 24 h of incubation.

Characterization and identification of bacteria

The selected bacterial strain *Bacillus flexus* RMWW II was characterized as rod-shaped, Gram-positive, oxidase and catalase-positive bacteria (Table S1, Supplementary Material). The complete details of the biochemical and physiological characteristics of bacterial strain *Bacillus flexus* RMWW II are given in Table S1. Partial 16S rDNA sequencing results obtained from Samved Biotech, Ahmedabad, India, revealed *Bacillus flexus* RMWW II. As shown in Figure S1 in the Supplementary Material, phylogenetic analysis demonstrated that the strain *Bacillus flexus* RMWW II clustered closely with *Bacillus flexus* 3S11 (Table S3, Supplementary Material). The selected isolate was identified as *Bacillus flexus* (GenBank accession no. KM374754.1). The variation in peaks obtained from HPLC data of control and bacterial degraded lignin might be due to the formation of new metabolites, confirming the degradation potential of the bacterial strain.

Alkali lignin biodegradation and associated growth dynamics of *Bacillus flexus* RMWW II

Impact of the initial concentration of alkali lignin

AL biodegradation potential of *Bacillus flexus* RMWW II at various concentrations (25–400 mg L^{-1}) in MSM at the end of nine days of incubation was determined. At the initial concentrations of 25 and 50 mg L^{-1}, the biodegradation efficiency was 100%. AL was the sole carbon and energy source in the inorganic medium that the bacterium had to consume for cell growth maintenance and survivability. The significant biodegradation efficiency at the initial concentrations is attributed to the abundant biomass that effectively utilized the AL as a carbon and energy source. Further, an increase in AL concentration from 100 to 400 mg L^{-1} witnessed a decline in the AL degradation from 97.1 to 20.15%. (Figure 2(a)). The bacterium was affected adversely by the toxic pollutant at higher concentrations. The high molecular weight and low solubility of lignin along with the increase in its concentration resulted in low degradability by bacterial action, which pertained to the substrate's inhibitory effect on the bacteria that subsequently influenced

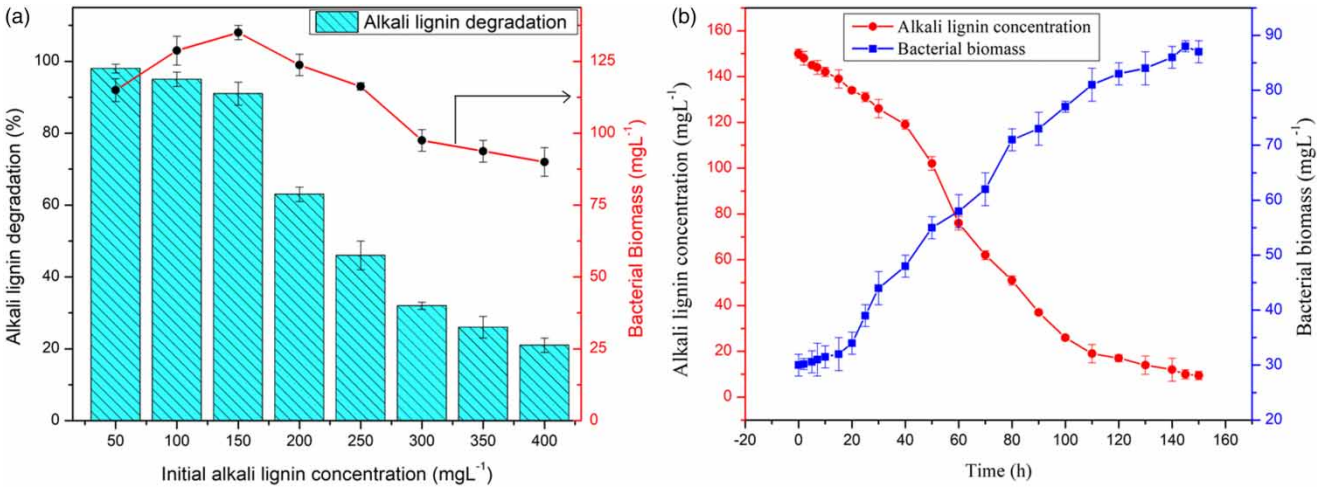

Figure 2 | AL degradation and *Bacillus flexus* RMWW II growth at (a) different initial concentrations of AL determined at the end of 9 days of incubation and (b) time course for AL biodegradation by *Bacillus flexus* RMWW II grown in MSM amended with 150 mg/L of AL as a sole source of carbon and energy.

the biodegradation of the strain. Similarly, the effect of incubation time on biomass and substrate concentrations of 150 mg L^{-1} is shown in Figure 2(b). There is a short lag phase after which there is a faster depletion of AL. At the end of 6 days, the AL reduction was 85%.

Kinetics of alkali lignin biodegradation

At various concentrations of AL (S), specific growth rate (μ) was plotted by the nonlinear curve, as shown in Figure 3(a). Values of K_s and K_i were found to be 65.1 mg L^{-1} and 296.5 mg L^{-1}, calculated using the Aiba model, which was suitably fitted with the experimental data having the lowest error ($SSE = 7.43 \times 10^{-7}$). The maximum specific growth rate (μ_{max}), true maximum specific growth rate (μ^*) and S_m were 0.0087 h^{-1}, 0.017 h^{-1}, and 138.93 mg L^{-1}, respectively. Appreciable values of K_i advocated the high potency of *Bacillus flexus* RMWW II, which signified the wider tolerability of the selected bacteria towards varied AL concentrations (Christen *et al.* 2012).

Three kinetic models were employed to determine the values of specific degradation rate q at each concentration (S) of the AL as shown in Figure 3(b). The Edward model gave the best fit with an SSE of 5.13×10^{-5} and the measured values of the specific degradation rate (q_{max}) and true specific degradation rate (q^*) equal to 0.056 h^{-1}

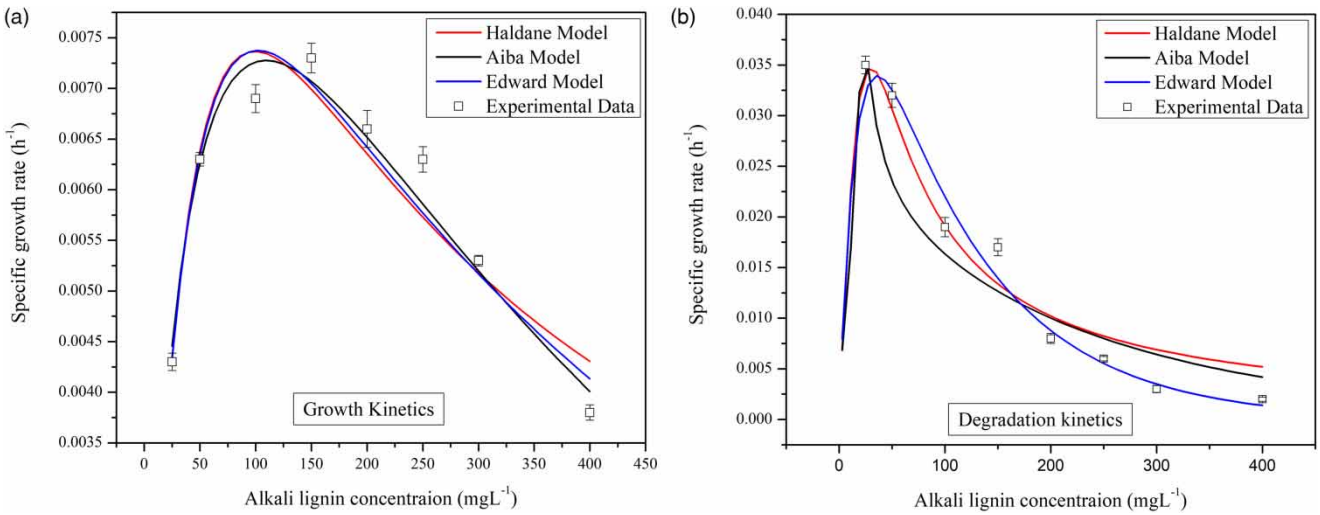

Figure 3 | Variation of (a) specific degradation rates and (b) specific growth rate of AL by *Bacillus flexus* RMWW II showing the closeness of fit of kinetic models (Haldane, Aiba and Edward model) with the actual experimental data.

and 0.042 h^{-1}, respectively. Increased concentrations of AL governed the values of the specific degradation rates (q, q_{max}, and q^*), which assisted in establishing the link between q and S by using substrate inhibition models. Table 1 summarizes various biokinetic parameters (K_S, K_i, K'_S, and K'_i) employing different models. The value of S'_m for q was observed to be 5.26 mg L^{-1}. Figure 2(a) showed that q_{max} and μ_{max} occurred at low initial AL concentrations (S_o) and upon further increase of (S_o), μ and q values significantly reduced. A comparison of kinetic constants obtained from this work to others obtained from the literature has been listed in Table S3.

A general trend of increase in degradation capacity and cell mass concentration with the increase in substrate concentration until a certain value and then a decrease with further substrate concentration increase (Figure 3(a)) indicates substrate inhibition on the bacterial activity. However, this inhibition is not very pronounced, as indicated by the high inhibition constant (K_i) in Table 1. The K_i indicates the maximum initial concentration of lignin which the bacteria can tolerate. The K_i values obtained in these studies are comparable to or higher than various other studies on degradation of lignin (Table S3), which indicates the possibility of using this bacteria even with higher initial substrate concentration. Although the values of μ_{max} and q_{max} are less, fairly good values of inhibition coefficient (K_i and K'_i) signified that the degradation rate is feebly inhibited at escalated concentrations (Christen *et al.* 2012). S_m values indicate the substrate concentration below which the growth is inhibited because of inadequate substrate and it also represents the substrate concentration above which the bacterial growth is inhibited due to excess substrate. A high value of S_m, which is pretty close to the usual initial concentration of lignin in rice mill wastewater, indicates that *Bacillus flexus* RMWW II can be used for lignin degradation in this kind of wastewater. From Figure 2(b), we can conclude that even at high initial substrate concentration, the initial lag phase is fairly short, indicating ease of using this bacteria for wastewater treatment as the bacteria starts degrading the contaminant within a short time after it comes into contact with it. This would allow us to design smaller vessels even with large wastewater volumes, hence reducing the capital investment. All these conditions make this bacterial strain a suitable candidate for lignin degradation and in turn remediation of rice mill wastewater even at escalated values of initial COD.

Paenibacillus sp., *A. aneurinilyticus,* and *Bacillus* sp. isolated from the sludge of a pulp and paper mill demonstrated the ability to degrade 100 mg L^{-1} of AL by 37%, 33%, and 30%, respectively with additional co-substrate requirements to arouse bacterial growth (Chandra *et al.* 2007). *Pseudomonas* genera were stated to biodegrade only 13% and 20% of AL in two separate studies. Recently, Wang *et al.* (2013) revealed a bacterial consortium designated as LDC that could degrade 60.9% of the lignin present in the reeds' structural composition. White-rot fungi such as *P. chrysosporium* are widely investigated for microbial bio-delignification. In a study reported by Janshekar *et al.* (1982), *P. chrysosporium* demonstrated 61% delignification at the end of 20 days with ammonium tartrate as co-substrates. The practical suitability of fungal delignification is often stifled by the need for additional expensive growth substrates. The complicated structure of fungal mycelia usually obstructs the machinery pipelines and also increases the bio-sludge generation, thus turning the whole process expensive. The concomitant kinetic details of the biodegradation process were not discussed in all the above-mentioned studies, which are prerequisites for scale-up.

Table 1 | Biokinetic parameters for biodegradation of AL by *Bacillus flexus* RMWW III determined by various models; (a) growth kinetics (b) degradation kinetics

(a)

Model	μ^* (h^{-1})	K_s (mg L^{-1})	K_i (mg L^{-1})	μ_{max} (h^{-1})	S'_m (mg L^{-1})	SSE
Haldane	0.024	108.1	91.2	0.008	99.29	1.02×10^{-6}
Edward	0.002	37.9	128.7	0.0018	7.41	8.63×10^{-7}
Aiba	0.017	65.1	296.5	0.0087	138.93	7.43×10^{-7}

(b)

Model	q^* (h^{-1})	K_s (mg L^{-1})	K_i (mg L^{-1})	q_{max} (h^{-1})	S'_m (mg L^{-1})	SSE
Haldane	0.048	245.8	0.423	0.058	4.91	6.64×10^{-5}
Edward	0.042	16.14	108.35	0.056	5.26	5.13×10^{-5}
Aiba	0.061	30.407	88.174	0.093	29.79	6.43×10^{-5}

FTIR spectroscopy study

FTIR analysis of control and bacterial-degraded AL samples was performed to explore the alterations in the functional group arrangement in the structure of AL due to the bacterial action after 7 days of the treatment period. Figure 4 depicted the FTIR spectra with major peaks around 1,100 cm^{-1}, 1,510 cm^{-1}, 1,600 cm^{-1} and 3,500 cm^{-1}. Peak shift at 1,115 cm^{-1} in the treated and untreated lignin indicated the presence of aromatic C–H coupled with syringyl fraction. The change in intensity of peaks at 1,602 cm^{-1} and 1,513 cm^{-1} indicates the destruction of certain aromatic rings and C–H bonds in the methoxy groups of lignin. However, there is no major shift of peaks at 1,285 cm^{-1}, which marks the Guaiacyl groups of lignin. The difference in peak position at 1,510 cm^{-1} in untreated to 1,515 cm^{-1} in the treated lignin represents the aromatic skeleton vibration (Casas *et al.* 2012). FTIR spectra further revealed that during lignin biodegradation, a strong increasing intensity at the 1,688–1,708 cm^{-1} band was collected in the bacterial degraded lignin, evidencing the generation of both conjugated as well as unconjugated carbonyl (C = O) groups (Liu *et al.* 2018). A marked difference was observed in the fingerprint region that lies between 3,700 and 3,400 cm^{-1}. The intensity of the O–H stretching vibration at 3,500 cm^{-1} sharply increases and no such absorbance was found in the untreated lignin (Kumar *et al.* 2016b). The O–H stretching suggested the oxidation of the aliphatic chain in the phenyl propane chain. This indicates the bacterial-mediated oxidative reaction on the complex structure of lignin that might lead to the destruction of the aromatic skeletal carbon of lignin, resulting in the absence of many bands present in the untreated lignin. The change in intensity around 1,100 to 1,400 cm^{-1} indicates cleavage of the β–O–4 linkage. This is the general route for phenolic compound formation from the degradation of lignin.

Phytotoxicity

In the present study, *Vigna radiata* seeds were employed as a bioindicator, which is one of the fast, sensitive and commonly cultivated pulses in the agricultural land of the Indian subcontinent. Liquid chromatography mass spectrometry (LC-MS) studies indicate the presence of alcohol like 2-butanol, 3-pentanone, and aromatics like *p*-cresol, 2 methoxy phenol. These compounds at very low concentrations do not attribute any toxic nature to the degraded wastewater. Root and shoot lengths of *V. radiata* seeds germinated in untreated and bacterial treated effluents were assessed and compared with the reference control. Percentage length at different concentrations (100–250) mg L^{-1} of AL was demonstrated (Figure S2, Supplementary Material). At the lignin concentration (200 to 250 mg L^{-1}), the seeds did not show good germination. For the roots, at higher lignin concentration (varying from 100 mg mL^{-1} to 250 mg mL^{-1}) the root length inhibition was 28% to 70%, while after treatment of lignin solution of the same concentration the inhibition dropped to 4% to 30% for the lowest

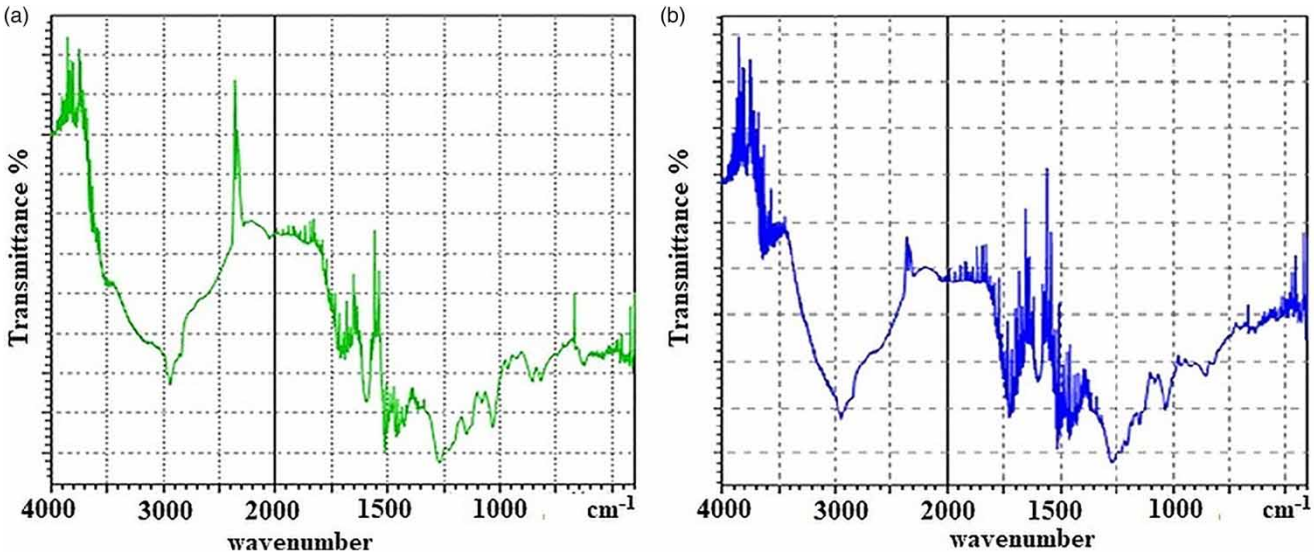

Figure 4 | FTIR analysis of (a) lignin (control) and (b) bacterial degraded AL samples after 7 days of degradation, indicating the formation of carbonyl bonds and hydroxyl bonds formed due to bacterial degradation.

and highest concentration respectively. A similar trend was seen for the shoot length also. Similar results were observed in other studies that give direct evidence for lignin degradation due to bacterial degradation (Majumdar *et al.* 2018). With further exposure at increasing concentrations, a high percentage of inhibition in lengths of root and shoot length was observed. The treated wastewater showed improved shoot and root growth when compared to the untreated counterparts. The toxicity study indicated the necessity for the treatment of untreated samples for water reuse for agricultural purposes.

Rice mill wastewater treatment

Bacterial-mediated bioremediation of rice mill wastewater was carried out in a batch experiment. The growth pattern of *Bacillus flexus* RMWW II revealed that the initial incubation phase has a short duration of lag phase with less noticeable changes in the COD content. However, as the growth of the bacterium reached log phase, rapid depletion in the value of COD occurred. A significant COD reduction of 84% was witnessed at the end of 70 h (Figure 5). Thereafter, a stationary phase began, where not much change was observed either in bacterial growth or COD values. The results will lead towards higher scale bioremediation of rice mill wastewater. This study combines the detail of biodegradation of AL as well as the removal of COD of rice mill wastewater. This is an added advantage in enhancing the understanding of the reduction of AL individually and when present in combination with other refractory compounds, in terms of COD removal from the wastewater.

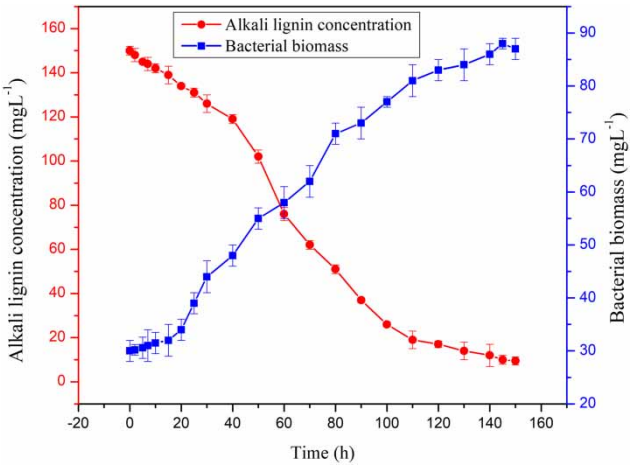

Figure 5 | Rice mill wastewater treatment in a batch study showing growth of bacterium and COD removal with time.

CONCLUSION

The present study illustrated an important novelty with the treatment of raw rice mill wastewater by *Bacillus flexus* RMWW II isolated from rice mill wastewater. The newly isolated bacterium showed biodegradation of complex AL, employing it as the sole carbon source. The biokinetic growth parameters were analyzed with various kinetic models and among the models tested, the Aiba model was found to fit the data well with a good correlation coefficient. The phytotoxicity was eventually evaluated and it was observed that bacterial-degraded samples were less toxic than the untreated sample. An appreciable value of 84% reduction in COD of real rice mill wastewater was obtained in the batch operation within a short duration of 70 h. This would be interesting to explore in the scale-up study of *Bacillus flexus* RMWW II in designed culture conditions. The overall findings suggested that the isolated robust bacterium possesses promising features towards pollution abatement of AL, and other refractory compounds laden in rice mill wastewater.

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CONFLICT OF INTEREST

None.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this paper is available online at https://dx.doi.org/10.2166/wst.2020.005.

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