Decolorization of Pulp and Paper Mill Effluent by Indigenous Bacterium Isolated from Sludge

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Pulp and paper mill effluent is rich in lignocellulosic compounds, and lignin is the major colour-imparting constituent present in it. The present study deals with isolation of a potential bacterium for decolourization of pulp and paper mill effluent. A promising lignin-degrading bacterium RJH-3 was isolated from sludge of pulp and paper mill and identified as bacillus *subtilis subsp. inaquosorum strain* by 16s rRNA gene sequencing. During wastewater degradation study by batch mode, the isolate reduced 59.6% lignin content, 61.3% colour, and 64.1% COD after 144 h of incubation. The bacterium was able to degrade lignin and decolorize pulp and paper mill effluent to the permissible discharge limit within 48 h retention time (RT) during the reactor study. The isolate efficiently reduced lignin (45.1%), colour (48.8%), and COD content (59.1%) at 48 h RT from the pulp and paper mill effluent at alkaline pH (8.3±0.3).

Keywords: Pulp and paper mill; Lignin; Colour, COD; Effluent; Bacillus subtilis

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INTRODUCTION

The pulp and paper industry is categorized among the most polluting industries due to the production of dark brown coloured toxic effluent, which causes air, water, and land pollution. The release of untreated or partly treated effluent into the water bodies bring about serious problems for aquatic life forms (Kesalkar *et al*. 2012), thus these industries are long being questioned in the context of environment related issues globally. The effluent characteristics depend upon the type of raw material used and the process employed during pulping or paper making (Tripathi *et al*. 2013). Pulp and paper industries use lignocelluloses as raw material for pulping and paper making. Lignocellulose mainly consists of cellulose, hemicelluloses, and lignin. During pulping most of the lignin is separated from cellulose fibres to obtain a fibrous mat. Lignin is known as nature's plastic, as it is not easily degraded in nature (Bholay *et al*. 2012). The pulp and paper effluent along with lignin and its derivatives contains adsorbable organohalogens, volatile organic carbons, resins, fatty acids, BOD (biological oxygen demand) content, COD (chemical oxygen demand) content, and alkaline pH (Pokhrel and Viraraghavan 2004). High molecular weight chromophoric compounds make the effluent

detrimental for human health (Tiku *et al*. 2010). Lignin is the major colour-imparting constituent in the effluent. Indian pulp and paper industries release approximately 697 million m^3 of effluent annually (Kumar and Subramanium 2014). Bioremediation of such a large quantity of effluent is a major challenge for each pulp and paper mill. Various methods have been reported for bioremediation of pulp and paper mill effluent. Tarak *et al*. 2011 investigated the use of various oxidation processes such as UV/H_2O_2, UV, and photo-Fenton process for the paper mill effluent treatment, while Mahesh *et al*. 2006 have reported the electrochemical degradation of agri-based paper mill wastewater (black liquor). The efficiency of the electrocoagulation technique for paper mill wastewater treatment was assessed by Katal and Pahlavanzadeh 2011; Kumar *et al*. 2011. But physical and chemical methods of wastewater treatment do not find much application in the industries due to their high operational cost (Chang *et al.* 2004). Constructed wetland (CW) systems have also emerged as an alternative low cost and sustainable wastewater treatment systems (Choudhary *et al.* 2011). Various biological methods have also been acknowledged for their effectiveness in decolorizing pulp and paper mill effluent. Fungi have been reported for decolourization and bioremediation of pulp and paper mill effluent; *e.g.* Saritha *et al*. (2010) and Senthil kumar *et al*. (2014) have reported *Aspergillus niger,* and Tiku *et al*. 2010 have reported *Phaenerochaete chrysosporium* for bioremediation of pulp and paper mill effluent. But fungal enzymes work only at acidic pH (below 5.0) and low temperature; thus they cannot be used for bioremediation of effluent in effluent treatment plants since the pH of the pulp and paper mill effluent is generally high (above 6.0). Bacterial enzymes could be a better choice, as they are stable at alkaline pH and high temperature. Numerous bacterial strains have been communicated to have the potential for bioremediation of pulp and paper mill effluent such as Chandra *et al*. 2009 have reported bioremediation of paper mill effluent using *Pseudomonas putida*, *Citrobacter* sp., and *Enterobacter* sp. Chandra *et al.* 2011 have also reported decolorization of black liquor using potential bacterial consortium comprising Serratia marcescens (GU193982), *Citrobacter* sp. (HQ873619), and *Klebsiella pneumoniae* (GU193983). Still the search for potential bacterial isolates is on throughout the world as the use of bacteria for bioremediation of effluent is restricted to lab scale only. Thus the present study was designed to isolate potential aboriginal bacteria present in pulp and paper mill sludge and study its degradation ability for decolourization of pulp and paper mill effluent.

EXPERIMENTAL

Materials

Collection of sludge samples for bacterial isolation

Assuming that the indigenous microflora present in the sludge of pulp and paper mill effluent treatment plants (ETPs) is better acclimatized to work under harsh conditions such as high BOD and COD, alkaline pH, low oxygen and high lignin concentration, sludge samples were collected from various ETPs near Saharanpur (Uttar Pradesh) and Yamuna Nagar (Haryana). The samples were collected in sterile polythene

bags and stored in cold room at 4 °C until further use. The freshly prepared effluent was used throughout the study.

Chemicals and reagents

Analytical grade chemicals and reagents were used throughout the study. All the solutions were prepared in distilled water. All the testing was performed according to Standard Microbiological methods for the Examination of Water and Wastewater (APHA).

Methods

Effluent collection and preparation of lab feed

Throughout the study the wastewater used was synthetic wastewater known as lab feed. It was prepared daily in the lab by mixing C_D and E_{OP} stage wastewater in the ratio of 2:1 collected from pulp and paper mill situated in North India. Black liquor and 10% solution of starch were also added to provide additional colour and COD (Hooda *et al*. 2015). The mixture was diluted with tap water. The resulting lab feed had COD, colour, and AOX values 500±25 mg O_2/L, 1000±50 Pt-Co unit, and 15±1 mg/L approximately. The pH of the lab feed was maintained 7.0±0.2 using NaOH and H_2SO_4. Nutrients such as N and P were added to the feed as described by Reddy *et al*. (2005) as per the standard nutrients ratio COD:N:P = 100:5:1. Unautoclaved lab feed was used for degradation studies to make it equivalent to ETP conditions.

Enrichment of indigenous bacteria

Mineral salt medium (MSM) was prepared (Chandra *et al*. 2007) by adding (in g/l: Na_2HPO_4- 2.4, K_2HPO_4- 2, NH_4NO_3- 0.1, $MgSO_4$- 0.01, $CaCl_2$- 0.01, D-glucose- 10.0, Peptone- 5), and trace element solution- 1mL/L(Karn *et al.* 2015). The pH of the medium was adjusted to 7.5±0.1 using NaOH, and H_2SO_4. 100 mL MSM was taken in a 250 mL flask and 5 g sludge was added to it. MSM was supplemented with lignin (100-400 ppm indulin or black liquor) as carbon source and incubated for 7 days at 37±1ºC and 150 rpm in an incubator shaker. After a week, 10 mL sample was retransferred in fresh 90 mL MSM supplemented with lignin and incubated for 72 h. In the same way, 3 more successive transfer of enriched sample were done.

Bacterial isolation

MSM agar plates containing lignin as carbon source (100 to 400 ppm) were used to isolate indigenous ligninolytic bacteria present in the enriched sample. The enriched sample was serially diluted (10^{-5}) in autoclaved distilled water and then spread on MSM (pH 7.5±0.1) agar plates. Plates were incubated at 35±1ºC for 4-7 days. The bacterial colonies were selected based on colony morphology, elevation, colour, microscopic observations and gram staining. Repeated sub-culturing was done to purify the bacterial isolates. Pure cultures were preserved for future use.

Screening of ligninolytic bacterial isolation

Bacteria that secrete ligninolytic enzymes (lignin peroxidase, manganese peroxidase and laccase) have been reported for lignin degradation (Bugg and

Rahmanpour 2015). Thus, to select bacteria which secrete these enzymes, a selection protocol based on dye degradation method was used. Bacterial isolates were streaked on MSM agar plates containing Azure B 0.02g (100 mL)$^{-1}$, Phenol red 0.01g (100 mL)$^{-1}$, and Ramazol brilliant blue 0.04g (100 mL)$^{-1}$ for lignin peroxidase, Mn peroxidase, and laccase (Kiiskinen *et al*. 2004; Pangallo *et al*. 2007), respectively. The plates were incubated in an incubator at 37^{o}C and observed for zone of decolourization. The isolates showing positive results were further selected for wastewater degradation study.

Wastewater degradation study using batch method

20 mL LB broth (in g/L: Casein enzymic hydrolysate- 10, Yeast extract- 5, Sodium chloride- 5) was inoculated with a loopful of selected bacterial culture. The flask was incubated at 37^{o}C in an incubator shaker and after 48 h of incubation, the culture was centrifuged, and pellet was transferred to 100 mL freshly prepared lab feed. Flasks were incubated at a temperature of 37±1 °C at 150 rpm in an incubator shaker. A control flask containing same quantity of lab feed without any culture inoculation was also maintained at the same experimental conditions. Treated samples from the flasks were daily analyzed for lignin degradation, COD and colour reduction by following the standard protocol. Lignin degradation and colour reduction was analyzed as per the methods described by Chandra and Abhishek (2011). To measure colour and residual lignin, treated lab feed and control flask samples were taken and centrifuged at 8,000 g for 30 min. Pellet was discarded and supernatant was used for analysis. Supernatant was diluted 3 times and pH adjusted to 7.4±0.2. The absorbance was taken at 465 nm for colour reduction and at 280 nm for lignin degradation on a UV–visible spectrophotometer. The colour was measured as platinum cobalt unit and lignin as ppm.

Molecular characterization of the bacterial isolate

The genomic DNA was isolated, and the amplification of the 16s rRNA gene was performed using the universal primers (Narde *et al*. 2004) Forward primer: 5'-AGAGTTTGATCCTGGCTCAG-3' and Reverse primer: 5'- ACGGCTACCTTGTT-ACGACTT-3'. Amplified rDNA region were sequenced by automated DNA sequencer - 3037*xl* DNA analyzer from Applied Biosystems using BigDye® Terminator v3.1 cycle sequencing Kit (Applied Biosystems). Sequenced data was aligned and dendrogram were generated using Sequence analysis software version 5.2 from Applied Biosystems. Sequences were compared to the non-redundant NCBI database by using BLASTN with the default settings used to find the most similar sequence and were sorted by the E score. A representative sequence of 10 most similar neighbours was aligned using CLUSTAL W2 for multiple alignments with the default settings. The multiple-alignment file was then used to create phylogram using MEGA5 software.

Wastewater degradation study in reactor by semi continuous mode

Fabricated lab scale reactors having a capacity of 2 liters were used for semi-continuous reactor study. The degradation efficiency of the bacterial isolates was analyzed at different retention time (RT) under controlled agitation (200 rpm) and aeration (1 to 2 ppm O_2). The selected bacterial isolate was grown in LB media for 48 h and centrifuged in autoclaved centrifuge tubes. The supernatant was discarded, and

pellets were dissolved in lab feed and then added to the reactor containing 1 litre of lab feed. A control reactor, which contained only 1 litre lab feed, was also maintained under the same experimental conditions. All the reactors were maintained at a controlled aeration, agitation and a temperature of 37±1 °C throughout the study. The bacterial culture was allowed to acclimatize and grow along with the indigenous microflora (since lab feed is unautoclaved) in the reactors for 10 days.

After acclimatization period the efficiency of bacterial strains was evaluated at various RT as per the standard formula RT=*V*/*Q*, where *V* is the volume of feed measured in mL and *Q* is the flow rate measured in mL/h. To maintain the same RT, addition and removal of feed was done twice in a day by removing and adding 1/3 and 2/3 parts after 16 and 8 h, respectively (Fig. 1). Efficiency of bacterial strains was evaluated daily in terms of reduction in colour, COD and lignin w.r.t. control reactor was checked (Hooda *et al.* 2015).

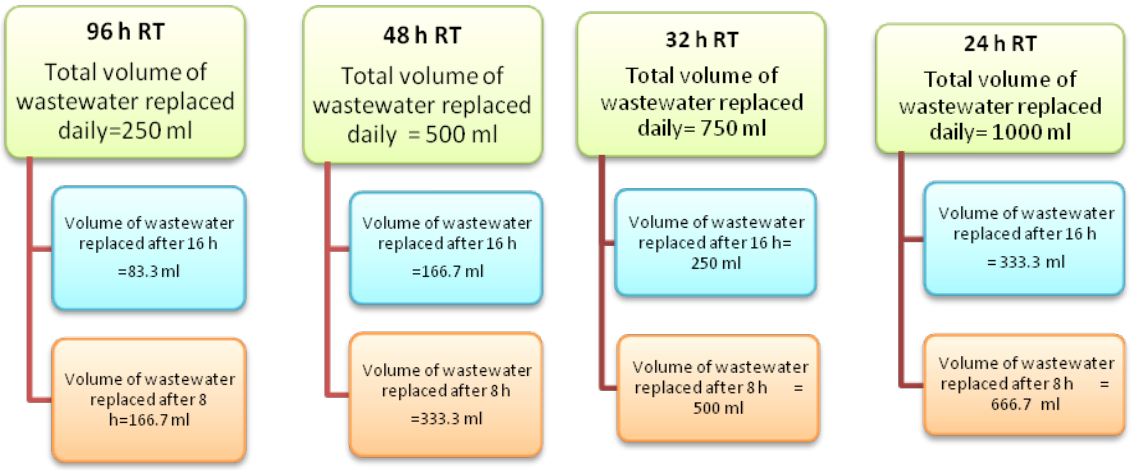

Fig. 1. Addition and removal of feed during reactor study

RESULTS AND DISCUSSION

Isolation and Screening of Ligninolytic Bacteria

After enrichment, 20 bacteria were isolated from sludge. After dye decolorization assay, 8 bacterial isolates were screened as positive for ligninolytic enzymes (Table 1).

Wastewater Degradation Study by Batch Method

Bacterial isolates positive for ligninolytic enzymes were evaluated for their ability to reduce colour, COD, and lignin of the lab feed (equivalent to pulp and paper mill wastewater), individually by a flask level study. RJH-3 was found to be the most efficient in remediation of the wastewater to the level permissible for safe discharge.

Table 1. Lab Isolates Showing Positive Results for Ligninolytic Enzymes during Dye Degradation Plate Assay

Lab isolate	Lignin peroxidase (Azure B)	Mn peroxidase (Phenol Red)	Laccase (Ramazol brilliant blue)
RJH-3	+	-	+
RJH-10	-	+	+
LAC-2	-	+	+
LAC-3	+	-	+
LAC-4	+	+	+
LAC-5	-	+	+
LAC-10	-	+	+
LAC-12	+	-	+

+ indicates presence of enzyme/ zone of decolourization,
- Indicates absence of enzyme/ zone of decolourization

During the 144 h batch study, the rate of degradation increased with increase in incubation time. Since the lab feed used for the study was not autoclaved, reduction was also observed in control flask by the indigenous bacteria present in the feed. When compared with the initial 0 h value of feed, control flask showed 3.5-13.6% lignin, 4.1-15.1% colour and 5.5-15.2% COD reductions after 144 h of the study.

The percent colour and COD reduction (Figs. 2 and 3) by RJH-3 w.r.t. control flask was 8.5, 15.9 after 24 h; 15.3, 32.4 after 48 h; 39.7, 37.6 after 72 h; 47.1, 50.2 after 96 h; 60.2, 63.4 after 120 h, and 61.3, 64.1 after 144 h, respectively.

The percent lignin degradation by RJH-3 after every 24 h was 6.8, 19.2, 42.1, 55.3, 58.1 and 59.6 (Fig. 4). Thus we conclude that rate of reduction increased continuously up to 120 h and afterwards little reduction was observed. This reduction may be associated with decrease in bacterial metabolism due to accumulation of toxic substances in the medium. Lignin is mainly responsible for dark colour of the effluent. So, when lignin content decreased, colour reduction was also observed as is evident from the results.

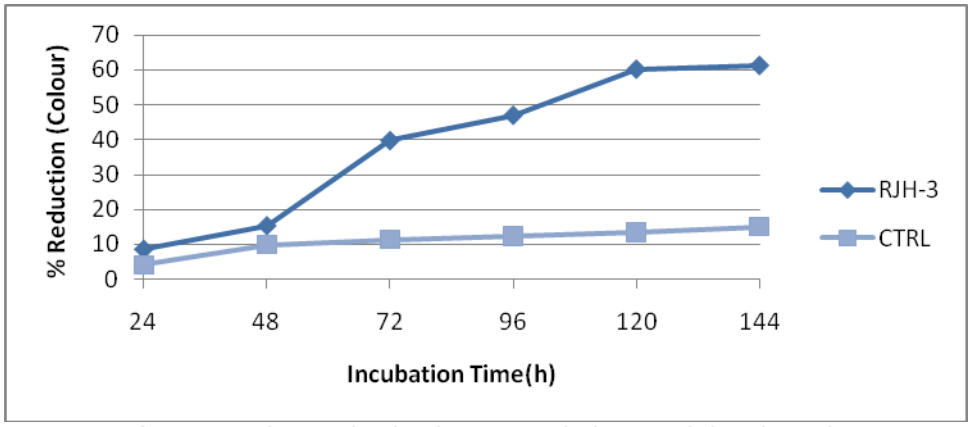

Fig. 2. % colour reduction by RJH-3 during 144 h batch study

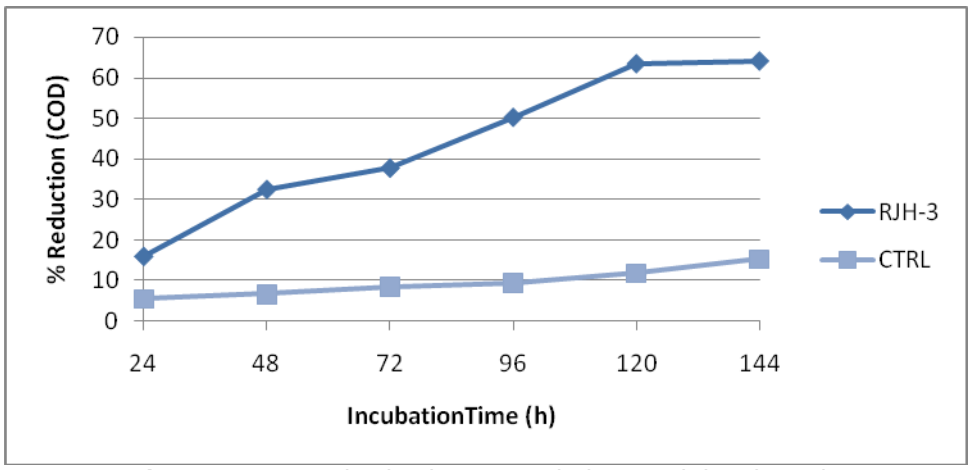

Fig. 3. % COD reduction by RJH-3 during 144 h batch study

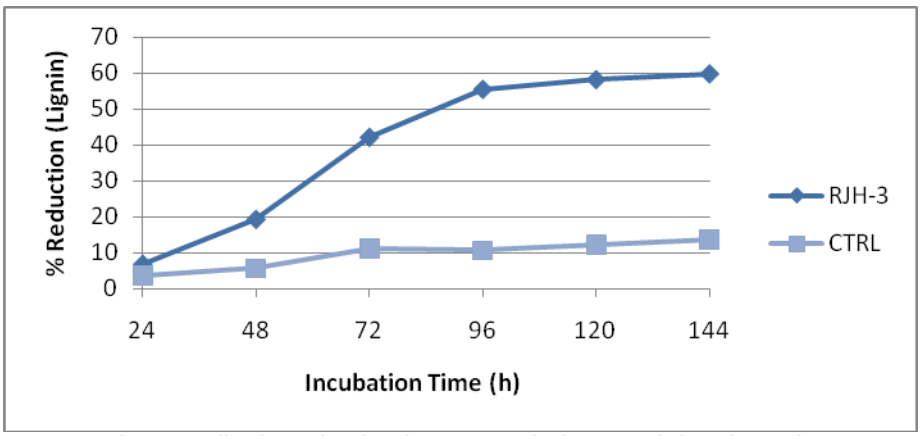

Fig. 4. % lignin reduction by RJH-3 during 144 h batch study

Molecular Characterization

16s rRNA gene sequencing revealed that the bacterial isolate RJH-3 is *Bacillus subtilis subsp. inaquosorum* strain. It showed 99% similarity to *Bacillus subtilis subsp. inaquosorum* strain BGSC 3A28, *Bacillus subtilis subsp. subtilis* strain DSM 10 and *Bacillus vallismortis* strain DSM11031. The isolate showed 98% similarity with *Bacillus amyloliquefaciens* strain NBRC 15535 and *Bacillus atrophaeus 1942* strain 19.

Wastewater Degradation Study in Reactor by Semi Continuous Mode

Wastewater degradation efficiency of RJH-3 was further assessed in reactor study by reducing the RT from 96 h to 24 h (Figs. 5, 6, 7). At 96 and 48 h RT the percent reduction in lignin, colour and COD was 45.3, 47.6, 57.6 and 45.1, 48.8, 59.1 while the same at 32 and 24 h RT was 39.7, 31.5, 56.8 and 25.5, 24.1, 25.1, respectively. The reduction was also observed in control reactor by indigenous bacterial population (Table 2). During the reactor study the pH reached to alkaline level and remained in the range of 8.3±0.3 as an outcome of bacterial metabolism. The results of the study conclude that RJH-3 is a potential contender for bioremediation of wastewater generated by pulp and paper mills.

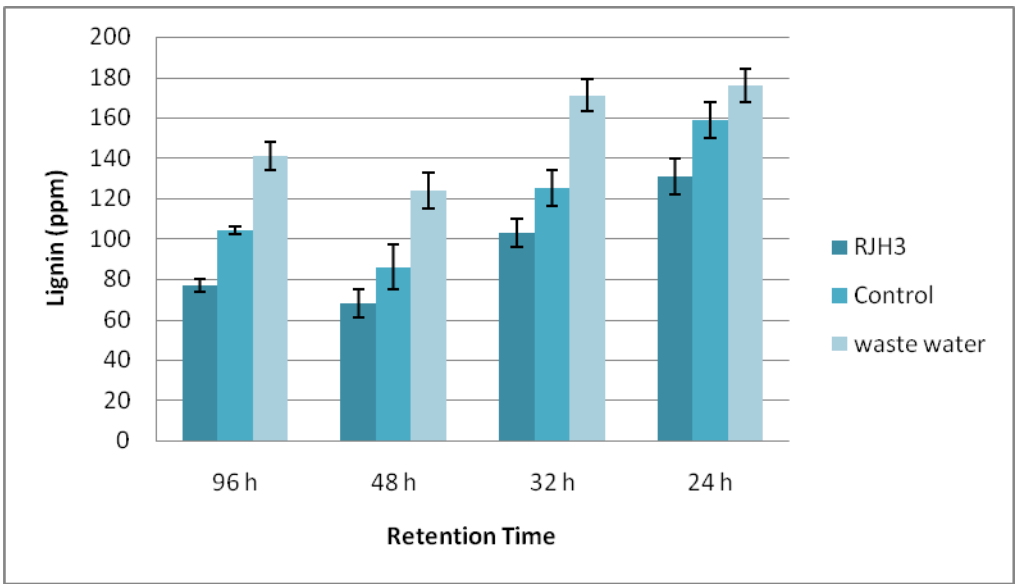

Fig. 5. Lignin degradation by RJH-3 during semi continuous reactor study

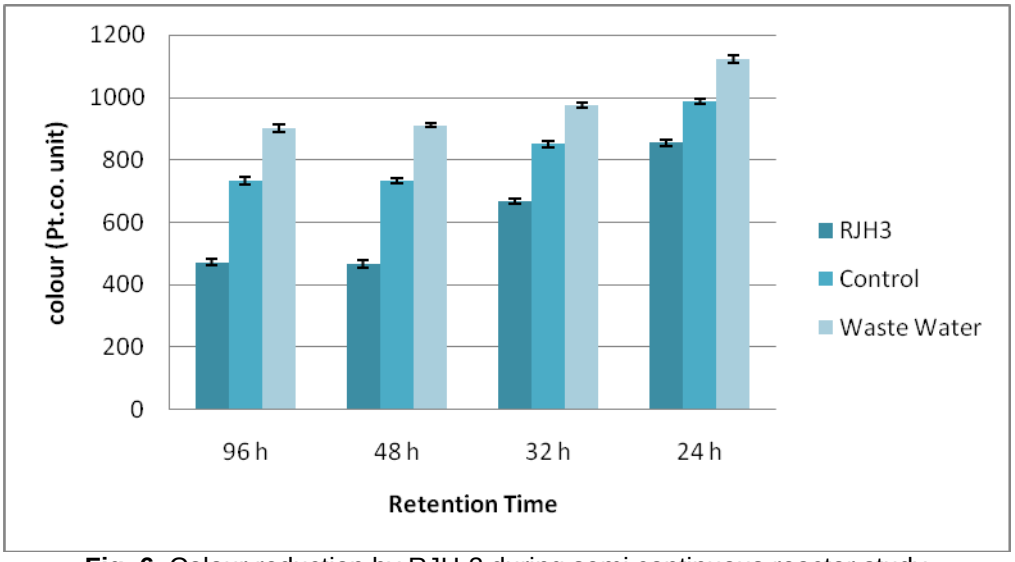

Fig. 6. Colour reduction by RJH-3 during semi continuous reactor study

The results were compared with the previously reported wastewater bioremediation experiments, in terms of incubation period, COD, colour and lignin reduction. Sharma *et al.* (2013) have reported that COD reduction decreases with increase in pH. Raj *et al.* (2007) have reported 61% colour, 53% lignin, and 78% COD reduction by *Bacillus sp.* after 6 day incubation. Similarly, Gupta *et al.* (2001) have reported the ability of *Aeromonas formicans* to reduce 70 to 80% of COD and lignin, whereas approx. 85% colour removal was achieved in 8 days. Chandra *et al.* (2009) demonstrated the use of *Bacillus cereus* and *Serratia marcescens* to reduce 45 to 52% of colour, 30 to 42% of lignin, 40 to 70% BOD, and 50 to 60% of COD in a 7-day period in the presence of glucose and peptone in the medium. Tyagi *et al.* (2014) used *B. Subtilis* and *M. luteus* and reported reduction up to 61.5%, 56.1 % and 94.7%, 89.6% in BOD and COD in 9 days,

respectively without supplementing additional carbon and nitrogen source. Here, in the present study we are reporting 45.1% lignin, 48.8% colour and 59.1% COD reduction by RJH-3, at a RT of 48 h without adding glucose or peptone as additional nutrient source which makes the process cost effective.

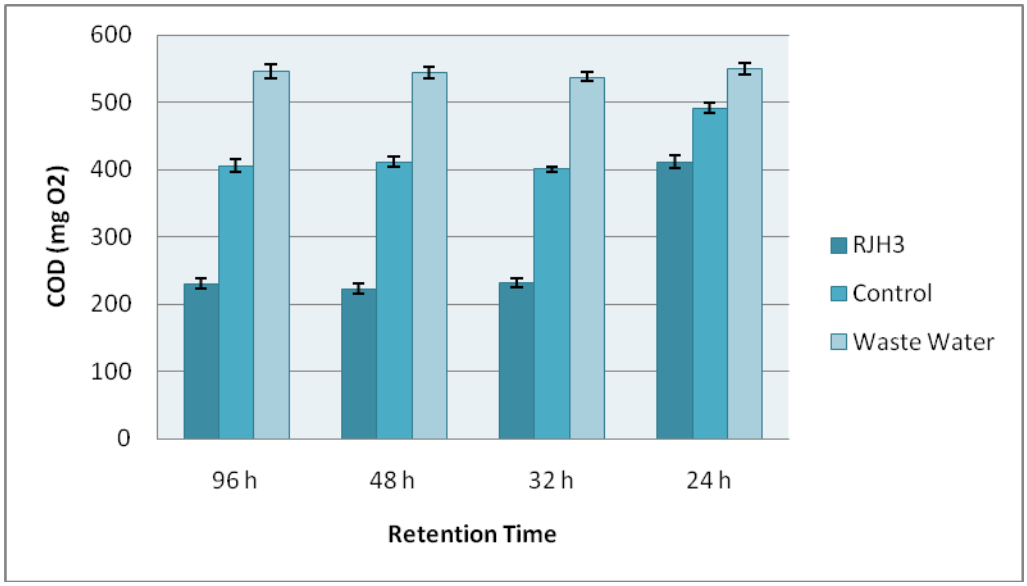

Fig. 7. COD reduction by RJH-3 during semi continuous reactor study

Table 2. COD, Lignin and Colour Reduction by RJH-3 during Reactor Study

RT (h) ↓	Lignin degradation (%)		Colour reduction (%)		COD reduction (%)	
	Control reactor	Reactor containing RJH-3	Control reactor	Reactor containing RJH-3	Control reactor	Reactor containing RJH-3
96	26.2	45.3	18.9	47.6	25.6	57.6
48	30.6	45.1	19.5	48.8	24.2	59.1
32	26.9	39.7	12.8	31.5	25.4	56.8
24	9.6	25.5	12.1	24.1	10.5	25.1

CONCLUSION

The results of the study showed that indigenous bacterium isolated from pulp and paper mill sludge is capable of using lignin as the sole carbon source and reducing the chemical oxygen demand (COD) and colour of the effluent at 48 h RT to a level safe for discharge into the environment. Since bioremediation of lignin rich pulp and paper mill effluent has emerged as a challenge for the environmentalists, the bacterium can play a lead role due to its significant bioremediation potential.

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