

Biological Approach for the Treatment of Pulp and Paper Industry Effluent in Sequence Batch Reactor

Virendra Kumar^{1,2}, Purnima Dhall¹, Sanjay Naithani², Anil Kumar^{3*} and Rita Kumar^{1*}

¹Institute of Genomics and Integrative Biology, Mall Road, Delhi, India

²Forest Research Institute, P.O. New Forest, Dehradun, India

³National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi-110067, India

Abstract

Pulp and paper industrial effluent is rich in recalcitrant compounds and causes pollution. For the treatment of such compounds activated sludge process is frequently used in which F/M ratio is kept low. This treatment results in effective biochemical oxygen demand removal but other waste water parameters are not reduced effectively due to lack of dissolved oxygen.

In the present study sequential batch reactor was used for the removal of pollutants from the waste water of pulp and paper mill by using bacterial consortium (*Klebsiella sp.*, *Alcaligenes sp.* and *Cronobacter sp.*). The aim of present research is to identify the influences of F/M ratio and dissolved oxygen concentration on the microorganism's growth and pollutant removal. The process of bioremediation was optimized by Taguchi approach. Bioremediation experiment resulted in reduction of chemical and biochemical oxygen demand up to 72.3% and 91.1%, respectively. A significant reduction in colour (55%), adsorbable organic halides (45.4%), total dissolved solids (22%) and total suspended solids (86.7%) was also observed within 14hrs while, the sludge volume index was 52. The wastewater after the treatment process meets the standard given by regulatory agencies and can be discharged into the environment without any risks.

Keywords: Adsorbable organic halide; Dissolved oxygen; F/M ratio; Sequential batch reactor

Introduction

An enormous industrial growth has taken place throughout the world in the past few decades. It has become so vast that, the environment has totally changed from what it was earlier. Due to increasing human needs, the level of pollution in environment has raised to devastating extends leading to disastrous consequences. Pollution today is found in each and every thing that we need the most viz. air, water, soil, etc. Water being one of the most important natural resources, is required in huge amount to fulfill all human needs. Apart from personal usage, the amount of water utilized by various industries is very large. Pulp and paper mills are one of the main water and energy intensive industries as it is sixth largest water polluting sector [1]. Typically in India around 75% of total fresh water supplied to pulp and paper industries emerges as waste water. In comparison to other industries fresh water requirement in pulp and paper industry is quite high (150-200 m³ per ton of product [2].

The problems associated with pulp and paper mill effluents are pH, colour, high levels of Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Suspended Solids (SS), Adsorbable Organic Halides (AOX) etc. Paper manufacturing process release chlorinated lignosulphonic acids, chlorinated resin acids, chlorinated phenols (trichlorophenol, trichloroguaiacol, tetrachloroguaiacol, dichlorophenol, dichloroguaiacol and pentachlorophenol), and chlorinated hydrocarbon in the effluent, are the major contaminants formed in the effluent of pulp and paper mill [3-6]. Pollutants released from pulp and paper mills into the environment pose numerous problems and physiological impairment. Furthermore some compounds in the effluents are resistant to biodegradation and can bio-accumulate in the aquatic food chain. Due to the high chemical diversity of the organic pollutants in paper and pulp mill waste water, a wide variety of toxic effects on aquatic communities in recipient watercourses have been observed [1,7-9].

Pulp and Paper mill wastewater treatment is of immense concern for

the environment due to its after effects. Several studies for the treatment of pulp and paper wastewater were carried out by using physicochemical treatment methods, viz., titanium oxide oxidation systems [10,11], Fenton and photo-Fenton reactions [12-14] and chemical coagulation of lignin [15]. Being energy intensive and more expensive these methods were not implemented at industrial sites. Researchers are now more focused in using biological treatment process for the treatment of pulp and paper wastewater because physical and chemical processes are not capable of removing biological oxygen demand (BOD) and low molecular weight compounds [16].

The researchers are more focused on environmental friendly technologies for the treatment of wastewater. Therefore they use biological approach for the removal of contaminants from the effluent. The biological treatment processes able to reduce colour, COD, BOD, and toxic low molecular weight chlorinated lignin derivatives [17,18].

Pulp and paper industry uses conventional activated sludge treatment process in which they are using nonspecific microorganisms and they kept food/microbe (F/M) ratio low in the aeration tank. A plant operating at low F/M ratio spectrum is characterized by a high concentration of sludge. They are maintaining the effluent treatment

***Corresponding authors:** Rita Kumar, Institute of Genomics and Integrative Biology, Mall Road, Delhi-110007, India, Tel: 91-11-27662133; Fax: 91-11-27667471; E-mail: rita@igib.res.in

Anil Kumar, National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi, India, Tel: +91-11-26717106; Fax: +91-11-26742125; E-mail: anilk@nii.ac.in

Received December 11, 2013; **Accepted** February 19, 2014; **Published** February 25, 2014

Citation: Kumar V, Dhall P, Naithani S, Kumar A, Kumar R (2014) Biological Approach for the Treatment of Pulp and Paper Industry Effluent in Sequence Batch Reactor. J Bioremed Biodeg 5: 218. doi:10.4172/2155-6199.1000218

Copyright: © 2014 Kumar V, et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

plant with high mixed liquor suspended solids (MLSS) in order to buffer the BOD, pH, waste water composition or temperature. The manufacturing process depends upon the demand therefore the raw material changes accordingly, which in turn changes the ultimate pollution load in the wastewater. If the MLSS of the tank is high it can tolerate the sudden shock. This treatment results in effective Biochemical Oxygen Demand (BOD) removal but face several problems. Low F/M ratio leads inadequate food for the population of microorganisms and problem arises in maintaining the sufficient dissolved oxygen concentration. This ultimately leads to enhanced growth of filamentous microorganisms. However, such systems are generally less effective in removing colour, COD and chlorinated phenolic compounds [19]. Therefore, more advanced alternative biological wastewater treatment strategies will be required for the treatment of effluent holistically and meet the new and more stringent discharge limits set for paper industry effluent. Such new biological treatment technologies must be developed to degrade lingo-cellulosic compounds and halogenated chemicals in effluents that pose the greatest threat to human health.

Mill description: The mill selected for the study is 50 year old and has capacity of 200 ton/day of paper production, situated in western Uttar Pradesh, India (29.9640°N, 77.5460°E). Kraft pulping process is the initial step followed oxygen delignification after that the chemical bleaching, is carried out in 3 steps (i) chlorination (C) (ii) extraction (E) (iii) hypochlorite treatment (H). Each step is preceded and followed by countercurrent washing with fresh water. A total of 25,000 KLD of water is consumed for the entire process of pulping and paper making whereas, around 23,000 m³/day is discharged as wastewater.

In the present study sequential batch reactor was used for the removal of pollutants from the waste water of pulp and paper mill by using bacterial consortium having potential to degrade lingo-cellulosic compounds. The aim of present research is to treat the pulp and paper effluent holistically in environmental friendly manner and also to know the influences of F/M ratio and Dissolve Oxygen (DO) concentration on the microorganism's growth and pollutant removal.

Materials and Methods

Chemicals and consumables

All the chemicals were of analytical grade and procured from Merck. Kraft lignin (KL), Carboxy Methyl Cellulose (CMC), birch wood xylan was procured from Sigma Aldrich. All the solutions were prepared in milli-Q water and were preserved in SCHOTT duran bottle. Media were procured from Himedia and glassware's and plastic wares were purchased from Rivera Pvt. Ltd.

Waste water sample collection and characteristics

Waste water sample collection: The samples were collected in bottles with at least 2.5 cm empty at neck to facilitate mixing by shaking, before examination [20]. For the present study, effluent samples were collected from over flow of primary clarifier (containing combined effluent of whole process) of large scale paper mill situated in UP, India. For collecting the samples composite sampling was done. The samples were collected in clean, plastic jerry can (container). Samples were transported within 4hr under refrigerated conditions and stored in cold room at 4°C till further use.

Collection of soil sample: Samples were collected on 5th May 2010 from the industrial premises. Soil samples (3 in number) were collected from the upper layer (2 cm depth) of the soil where most of the microbial activity takes place. Three areas, Wood Yard Section (WYS), Combined Bleach Effluent discharged point (CBE) and Final Discharge

Point (FDP) were selected for the sample collection. From each area three random samples were collected. Those random samples were mixed to get one sample from each area. All the samples were collected in plastic bags. After collection the bags were kept at 4°C till further use.

Characteristics of waste water: The effluent was characterized for various physico-chemical parameters like: pH, color, lignin, COD, BOD, Total Dissolved Solids (TDS), Total Suspended Solids (TSS), AOX and each parameter was analyzed in triplicate in order to see the standard error in handling. All analyses were carried out as per standard procedures [20]. Color was measured by the spectroscopic method (National Council for Air and Stream Improvement-NCASI Method 71.01.1999) [21]. AOX of the effluents was measured using the procedure standardized in Germany in 1985 and known as DIN 38 409 Tail 14 AOX procedure.

Isolation of autochthonous bacteria

Bacteria were isolated from the pulp and paper mill premises. Three samples were collected (i) from wood yard section, (ii) combined bleach effluent discharged point, (iii) soil from near the final discharge point. The pulp and paper waste water is rich in lignin and other chemicals. The areas for collecting the soil samples were selected by assuming the presence of delignifying bacteria in that sector with recalcitrant compounds and genetic potency to degrade these compounds. Isolation was done by using such soil samples because it is easy to acclimatize those bacteria in the paper industry effluent due to their existence in particular environment and these bacteria can be enriched in the presence of toxic compounds therefore, a significant strains will be evolved with the process of acclimatization. Serial dilution of enrichment culture (supplementary Table 1) was done after 45 days of incubation at 150 rpm and 35°C. For each soil sample three enrichment flasks were prepared containing different substrate i.e., lignin, Carboxymethyl Cellulose (CMC) and birch wood xylan. Different media were prepared in order to isolate the bacteria which will able to degrade lingo-cellulosic compounds (supplementary Table 2).

For preparing the bacteria for the task, sub-culturing was done in the enrichment media after every 15 days for 3 times.

After serial dilution the 50 µl of the dilution was spread on the agar plates of minimal salt medium (MSM) containing lignin/cellulose/xylan as sole carbon and energy source. These plates were incubated at 35°C for 7 days and bacteria were purified at nutrient agar plates. Plates were preserved at low temperature till further use.

Screening of selected isolates

The screening method included functional assays for the presence of lingo-cellulosic compound degrading enzymes. Decolourization of lignin-mimicking dyes was assessed in agar plates. The plates were prepared by autoclaving the nutrient agar at 15 psi for 20 min. The following dyes were selected: AB: Azure-B; BB: Bromophenol Blue; MB: Methylene Blue; PR: Phenol Red; MG: Malachite Green. Dye was added individually 50 mg/l of media and poured in to the plates. After pouring, the plates were kept for solidification at room temperature. Sterilized disc borer was used to cut the disc of approximately 1 cm (dia) size in solidify plates.

Bacterial culture supernatant (200 µl) was poured into the well. The plates were incubated at 35°C and monitored daily for 3 days. Bacterial isolates which produced a visible change in the colour of substrate were selected for bioremediation study.

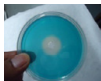




Exp. No.	P1	P2	P3	P4	P5	P6	Percentage reduction in COD			S/N Ratio
	Temp	pH	F/M	DO	Time	NP	T1	T2	T3	
1	25	6	0.5	0.5	14	100:6:1.5	55	60	58	27.2
2	25	6	1	1	16	100:5:1	67	65	67	28.41
3	25	6	1.5	1.5	18	100:4:0.5	57	61	59	29.83
4	25	7	0.5	0.5	16	100:5:1	68	65	72	25.77
5	25	7	1	1	18	100:4:0.5	65	69	71	26.98
6	25	7	1.5	1.5	14	100:6:1.5	59	65	61	26.09
7	30	8	0.5	1	14	100:4:0.5	71	65	69	26.98
8	30	8	1	1.5	16	100:6:1.5	64	67	67	28.6
9	30	8	1.5	0.5	18	100:5:1	71	67	63	24.47
10	30	6	0.5	1.5	18	100:5:1	64	69	72	24.54
11	30	6	1	0.5	14	100:4:0.5	69	71	74	29.04
12	30	6	1.5	1	16	100:6:1.5	64	69	64	27.13
13	35	7	0.5	1	18	100:6:1.5	72	67	70	28.84
14	35	7	1	1.5	14	100:5:1	74	73	76	33.74
15	35	7	1.5	0.5	16	100:4:0.5	58	69	64	21.23
16	35	8	0.5	1.5	16	100:4:0.5	69	71	64	25.5
17	35	8	1	0.5	18	100:6:1.5	72	69	74	29.09
18	35	8	1.5	1	14	100:5:1	65	68	71	27.1

Table 1: Results of trials carried out for different experiments designed using Taguchi approach and their S/N ratios.

	T(°C)	pH	COD (mgL ⁻¹)	BOD (mgL ⁻¹)	TDS (mgL ⁻¹)	TSS (mgL ⁻¹)	AOX (mgL ⁻¹)	Color (PCU)
Winter	20-29	7-8.5	600-1000	250-350	1100-1400	120-180	10-18	700-1200
Summer	25-36	7-8.5	550-900	200-300	1000-1300	90-150	8-16	600-1000

T-Temperature, COD- Chemical Oxygen Demand, BOD-Biochemical Oxygen Demand, TDS-Total dissolved Solids, TSS- Total Suspended Solids, AOX- Adsorbable Organic Halides.

Table 2: Characteristics of wastewater during summer and winter months.

Lab Name of Bacteria	MG	MB	AB	PR	TA
Plates showing positive results					
PNP1	+++	+++	+++	-	+++
PNP2	+	-	-	+	+
PNP3	+++	-	+++	+	+++
PNP4	++	+	++	+	+
PNP5	-	-	-	-	-
PNP6	-	+++	+++	+++	+++
PNP7	-	-	-	-	-
PNP8	++	+++	+++	+	+++
PNP9	-	-	-	-	-
PNP10	-	-	-	-	-

Note* +++ = Maximum; + = Minimum; - = No decolorization

Table 3: Decolorization of dyes by isolated bacteria.

Formulation and screening of bacterial consortia

Pulp and paper wastewater is complex and toxic in nature so, it is difficult for single microorganism to degrade it effectively. Therefore, selected single isolates were used for the formulation of different consortia. Each consortium consists of three bacteria. Total four consortia were formulated by random combination isolates (supplementary Table 3).

Different cultures were inoculated in 25 mL of nutrient broth (NB) and incubated overnight at 35°C and 150 rpm. The mother cultures were checked by streaking on nutrient agar plates which were then incubated at 35°C for 16-18 hrs. These mother cultures were used for sub culturing. 100 µl of culture was inoculated into 100 mL of NB and incubated at 35°C under shaking conditions for a period of

16-18 h. The culture was harvested by centrifugation at 4°C and 7000 rpm followed by washing twice with sodium phosphate buffer (pH 6.8-7.0). The supernatant was discarded and pellets were stored for the further experiments. At the time of experiment, different pellets were re-suspended according to the 4 consortia designed and inoculated in the wastewater of pulp and paper industry, the effluent sample was supplemented with nutrients N and P in the form of urea and diammonium hydrogen phosphate in the ratio of (BOD) 100: nitrogen (N) 5: phosphorus (P) 1 and the flasks were kept in shaking incubator at 150 rpm and 35°C for 24 h. After incubation, Chemical Oxygen Demand (COD) was estimated according to the procedure mentioned in standard methods, American Public Health Association (APHA). The obtained results were compared with the control flask which is

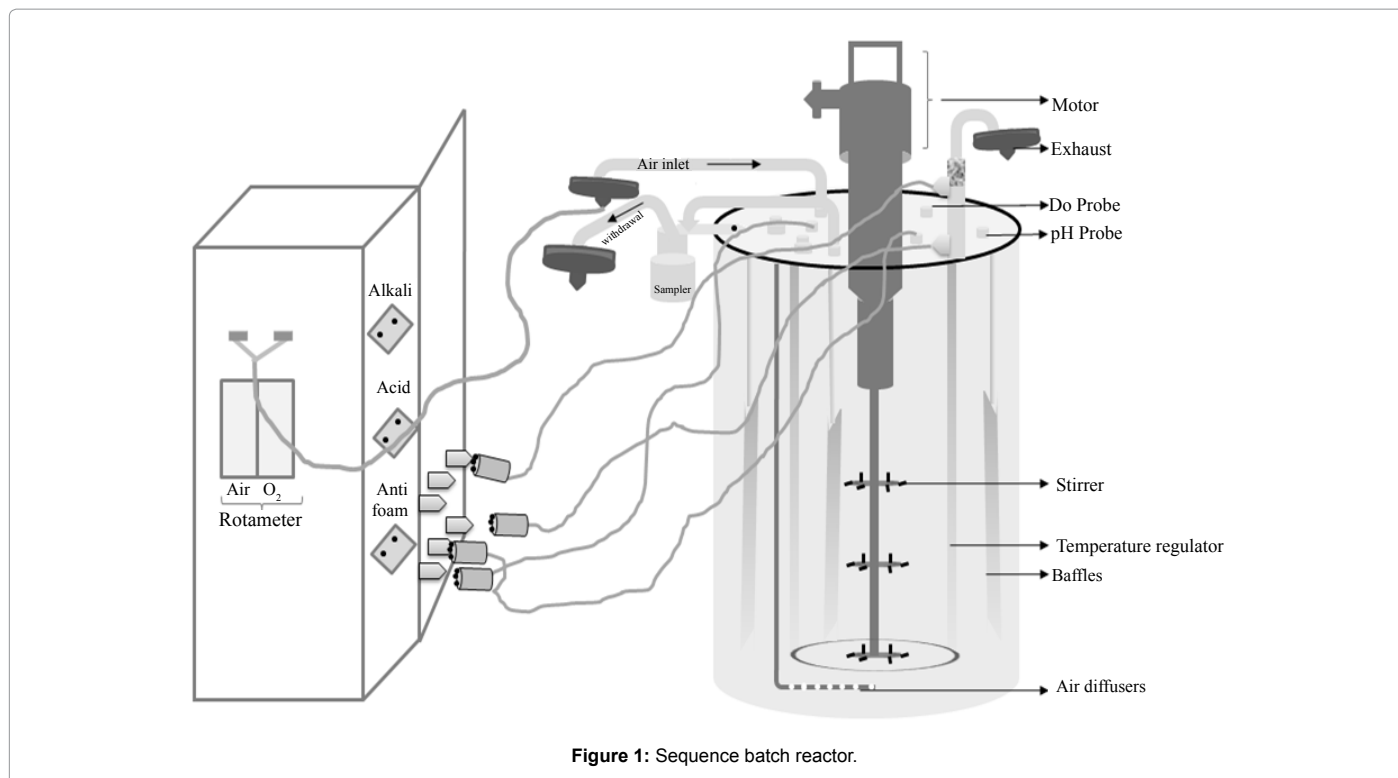


Figure 1: Sequence batch reactor.

incubated at same condition without consortia i.e., wastewater with the supplements.

Details of bioreactor

Laboratory scale sequence batch reactor (SBR) was used with a working volume of 12 liters having a glass vessel accompanying the baffles for minimizing the foam during the treatment process.

On the upper side of the metal lid there is one motor attached to the shaft on which blades were embedded for stirring inside the vessel. Different ports were provided one for air supply and two for filling and emptying vessel. On the surface of the metal lid probes for pH and DO were also entrenched.

For regulating the temperature electric blanket was provided. This blanket covers the vessel from outside. For decreasing the temperature inside the vessel water circulating coil was present which is attached to the chiller outside.

Air supply was provided by a compressor pump at 2 bar (30 psi) via a solenoid valve to a coarse bubble diffuser. Air flow was regulated by a rotameter. An exhaust tube was used on the surface of the lid of the reactor attached with 0.45micron filter. Air was passed through a filter of same size to remove impurities before it enters the reactor. Figure 1 depicts the details of the bioreactor.

Operation of SBR

Cycles involved during the operation: The SBR cycle consists of four steps (i) filling, (ii) hydraulic react, (iii) settling and (iv) decant. While working with the bioreactor

- i. First step involves filling of the vessel. For filling the vessel with wastewater peristaltic pump was used. Time required to fill the vessel was 10-15min approximately.

- ii. The bacterial isolates of selected consortium were grown separately. Cells were harvested (approximately 3.9×10^9 CFUml⁻¹) and inoculated in bioreactor. Initial inoculum was 10% i.e., 1L consortium pellet: 10L wastewater. After that hydraulic react i.e., the aeration and mixing of bacterial consortium with effluent was provided for 14-18hrs.
- iii. Followed by the settling time i.e., 1-2hr.
- iv. Then treated wastewater was decanted which take approximately 10-15min. The treated sample was analyzed for chemical oxygen demand. During the treatment MLSS was maintained by removing the extra sludge from the vessel by time to time after settling.

Optimization of treatment parameters in bioreactor

Taguchi approach: Taguchi method is a statistical method developed by Genichi Taguchi during the 1950s as an optimization process technique. Signal to noise ratio is the measure of performance as proposed by Taguchi. This ratio considers both the mean and the variability. The S/N measure the level of performance and the effect of noise factors on performance and is an evaluation of the stability of performance of an output characteristic.

The S/N is calculated as given in for larger the better

$$\frac{S}{N} = -10 * \log_{10} \left[1/n \sum_{i=1}^n 1/Y_i^2 \right]$$

Taguchi approach uses orthogonal arrays for designing the experiments and analysis of variance for generation of significant results. The importance of using this approach is to reduce the number of experiments [22-25].

The main aim to use the Taguchi approach is to reduce the number of experiments and get maximum output with selected optimum

parameters. Taguchi approach was used to optimize the six parameters each with three variables. The selected parameters were temperature (25-35°C), pH (6-8), F/M ratio (0.5-1.5), dissolve oxygen (0.5-1.5 mg/l), hydraulic react time of treatment (14-18 hrs) and nutrient dosing (NP) (BOD100:N4:P0.5-BOD100:N6:P1.5).

Experimental design: In this study six parameters at three levels were optimized. First parameter was **temperature** studied at 25°C (level 1), 30°C (level 2) and 35°C (level 3), second **pH** at 6 (level 1), 7 (level 2) and 8 (level 3), ***F/M ratio** at 0.5 (level 1), 1.0 (level 2) and 1.5 (level 3), **DO (mg/l)** at 0.5 (level 1), 1.0 (level 2) and 1.5 (level 3), **hydraulic react time** (hours) at 14 (level 1), 16 (level 2) and 18 (level 3), **nutrients (BOD: N: P)** at 100:6:1.5 (level 1), 100:5:1 (level 2) and 100:4:0.5 (level 3). Table 1 gives the details of eighteen experiments to be conducted according to L-18 orthogonal array. If we apply fractional factorial design the number of experiments required to optimize six parameters at three levels are 729 while they reduced to 18 by applying Taguchi approach. Taguchi approach will not only reduce the number of experiment but also indirectly help us to reduce the total expenditure in terms of time and cost.

Verification experiment: After applying the Taguchi approach to several parameters, primary result was obtained. In order to verify the obtained results repeatability of the experiment was done with the selected optimized parameters i.e. temperature (35°C), pH (7), F/M ratio (1), DO (1.5), hydraulic react time (14 hrs), nutrients (100:5:1). This step is essential for the verification of results obtained during the Taguchi approach.

Identification: The isolates of selected consortium were identified by using 16S rRNA gene sequencing. Bacterial DNA was prepared using bacterial DNA isolation kit from Real Genomics. The 16S rRNA gene was selectively amplified with the 16S universal primers. The primer sequences were, forward primer 5'AGAGTTTGATCATGGCTCAG3' and reverse primer 5'TACGGCTACCTGTTCAGACTT3' [23]. The PCR reaction composition consist of 50 µl of PCR reaction mixture containing 5.0 µl of 1.5 mM of kit buffer, 5.0 µl of template, 1.0 µl of 20 mM of dNTPs Mix, 1.0 µl of 20 mM of each primer, 1.0 unit of Taq polymerase and 37 µl of distilled water. Amplification of reaction mixture was done on DNA engine (BIORAD). Thermocycling protocol included initial denaturation of 94°C for 4 min followed by denaturation, annealing and elongation temperature of 94°C for 1 min, 52°C for 45 sec and 72°C for 1 min, respectively for 35 cycles. Final extension was done at 70°C for 8 min. Amplified products were run on 1% agarose gel and purified using the Qiaquick PCR purification kit (Qiagen, Valencia, CA, U.S.A.) and sequenced (Applied Biosystems Genetic Analyzer). All the amplified sequences were assembled using SeqMan program (Version 5.07, DNA Star, Inc.) to generate a consensus sequence which was then compared with all available nucleotide sequences using NCBI-BLAST to identify the strain. The nucleotide sequences of the 16 S rRNA genes of all the described species were retrieved from GenBank and aligned with the almost complete 16S rRNA gene sequence using ClustalW. Evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Kimura 2-parameter method. Phylogenetic analyses were conducted in MEGA 5.1. Confidence in the tree topology was determined by bootstrap analysis using 1000 replicates of the sequences.

Survival of the bacterial isolates: It is necessary to check that the isolates of our selected consortium is surviving or not in the effluent during and after the treatment process. Further, to confirm the presence of developed bacteria in the SBR, sludge samples were collected at different time interval. The survival of isolates in the effluent was tested

after successful completion of bioremediation cycles. The collected samples were appropriately serially diluted and the dilutions up to 10⁻¹² were spread on nutrient agar plates. The plates were incubated at 35°C for 16-18hrs. The colonies were counted by using colony counter.

Results and Discussion

Waste water characteristics

Pollution loading of the pulp and paper industrial wastewater depends significantly on the raw material used. The nature of the effluent is toxic due to the presence of various chemicals and lignin compounds present therein. Wastewater characteristics were analyzed in order to see the fluctuations in loadings during mill production process. This analysis will give us a brief idea of pollution load handle daily by the industrial persons. The waste water was collected over a period of time (1year). The effluent was characterized for various physicochemical parameters like: pH, color, lignin, COD, BOD, TDS, TSS, AOX and each parameter was analyzed in triplicate in order to see the standard error in handling. Results were depicted in Table 2. The results showed slight increase in pollution loading during winter than in summer. This might be due to, the temperature is more suitable for the indigenous bacteria to degrade the pollutant present in the effluent effectively and during rainy season the wastewater is diluted to some extent.

Isolation of autochthonous bacteria

Ten bacteria were isolated from 3 soil samples. The bacteria were characterized on the basis of its colony morphology. The morphological characteristics studies were color (yellow, white and cream), size (1 mm, 2 mm, pinpoint), elevation (concave, convex, flat), shape (irregular, smooth, zigzag).

Screening of isolated bacteria

Bacterial isolates were characterized for the presence of lignin degrading enzymes by dye decolorization agar plate method. The enzymes involved in lignin degradation (laccase, lignin peroxidase, manganese peroxidase) are also able to oxidize certain substrates like; ABTS, guaiacol, tannic acid and can decolorize some dyes such as azure – B, phenol red, malachite green, methylene blue. These compounds were used by lignin degrading enzymes as substrate analogue or nonspecific substrate because they structurally resemble lignin. Bacterial isolates which produced a visible change in the color of substrate were selected for treatment of wastewater. Ten isolates were screened and results were depicted in Table 3. PNP1 showed maximum decolorization for malchite green, methylene blue, azure B and tannic acid similarly, the PNP3 bacterium showed maximum decolorization for malchite green, azure B and tannic acid. Another two bacteria PNP6 and PNP8 showed maximum decolorization for methylene blue, azure B and tannic acid. PNP 6 also showed dye decolorization in phenol red. Therefore, these four bacterial PNP1, PNP3, PNP6 and PNP8 were selected for the formulation of consortia.

Formulation and screening of consortia

Mixed community can overcome the problems faced by single culture to treat the complex effluent. Therefore, different consortia were formulated from the selected isolates. Isolates were selected by screening their ability to decolorize the respective dyes and substrate mentioned above. To check the capability of the consortia i.e., the combination can act well in synergism or not the experiment was designed. The observations were made by analyzing the sample on the basis of chemical oxygen demand. This parameter was taken in to consideration because all other water pollution parameters were also

Consortia Lab name	Combination of isolates	COD mg/l Mean*	Percentage Reduction w.r.t Control	Standard Deviation	Satndard Error
Control		750		1.22	±0.55
1	PNP1, PNP6, PNP 8	350.6	53.25	0.89	±0.40
2	PNP3, PNP6, PNP8	309	58.8	1.58	±0.71
3	PN 3, PNP1, PNP8	375.8	49.8	2.59	±1.16
4	PNP1, PNP3, PNP6	451.6	39.7	1.82	±0.81

* each sample was analyzed five times by placing its COD.

Table 4: Screening of formulated consortia (n=5).

Level	P1	P2	P3	P4	P5	P6
1	27.38	27.69	26.47	26.13	28.35	27.82
2	26.79	27.1	29.31	27.57	26.1	27.33
3	27.58	26.96	25.97	28.05	27.29	26.59
ΔS/N	0.79	0.74	3.34	1.92	2.25	1.23
Rank	5	6	1	3	2	4

Table 5: Ranking of parameters based on the average S/N values.

decreased once the COD reduced effectively. Each sample was analyzed five times by placing the chemical oxygen demand in order to check the reproducibility among the results and also have a look on handling error. Results revealed that the consortium no. 2 was effective in the degradation by reaching the value up to 309 mg/l with the percentage reduction 58.8% with respect to other consortia. The standard error and deviation for the consortium 2 is ±0.71 and 1.58, respectively (Table 4). Therefore consortium 2 was selected for further studies.

Optimization of Parameter

Taguchi approach: L-18 array was used in which the total number of experiments were eighteen. After observing the results experiment no. 14 was selected giving the best results after running three times (T1-T3). Experiment no 14 with parameters- temperature 35°C, pH - 7, F/M ratio - 1, DO - 1.5, hydraulic react time- 14 hrs and Nutrients dose-100:5:1 showed the best result. The percentage reduction observed in COD after trial 1, 2 and 3 were 74%, 73% and 76% respectively (Table 1). The S/N ratios were calculated on the basis of the larger the better criteria. The effect of different parameters on the treatment was estimated by calculating the average S/N ratios for all the levels of every parameter and then estimating the difference between the maximum and the minimum S/N ratio (ΔS/N). The parameter with largest difference is expected to cause maximum impact on the process. Based on these results, the parameter F/M ratio had the maximum effect on treatment (Table 5). The analysis for S/N data at each level of each parameter is also plotted in graphical form (Figure 2).

Bioremediation approach was opted to treat the wastewater in environmental friendly manner. In this approach the bacteria utilized the oxygen to degrade the pollutants/organic matter present in the wastewater. During this process number of factors will directly or indirectly affect the growth of the microorganisms to complete the remediation process effectively [24]. Therefore in this study, temperature, pH, F/M ratio, dissolve oxygen, hydraulic react time of treatment and nutrients were selected as essential parameters. Temperature was selected because it greatly affects the activity of microbes [25]. There are different types of bacteria (Psychrophiles exist at 5°C-35°C, Mesophile at 25°C-40°C and Thermophiles at 25°C-75°C) depending on the temperature range. The treatment of wastewater is affected with the fluctuations observed in temperature. The range of temperature observed during the one year study of waste water characteristics fall between 25°C - 35°C. Therefore, the three levels were selected for temperature i.e., 25°C, 30°C, 35°C.

The next selected parameter was pH it ensures the healthy and effective system. The normal pH in wastewater for bacterial survival is 5 to 9. But, neutral pH i.e. about 6.5 - 7.5 is required for the optimum activity of the bacteria. If the pH is in acidic range fungi can become predominant than the bacteria which will results in poor settling. If the pH is too high it will affect the metabolic activity of the microbes which directly affects the treatment process [26]. For bacteria there is an orderly increase in growth rate between the minimum and the optimum and a corresponding orderly decrease in growth rate between the optimum and the maximum pH. Therefore the levels selected for the pH were within the optimum range. The selected levels were pH 6, 7 and 8.

The third parameter selected was F/M ratio. The food to microorganism ratio is defined as the ratio between the amount of food (organic matter) entering the treatment plant and the mass of micro-organisms (MLSS) in the aeration tank [27]. The F/M ratio is an important control parameter as the quantity of biomass present will influence the removal efficiency.

The F/M ratio is indirectly proportional to the MLSS. If F/M ratio is low, then MLSS is high with low dissolve oxygen concentration. Due to which filamentous bulking occurs that cause poor settling problem. If settling is not proper than amount of TSS increased in wastewater. If F/M ratio is high then, MLSS is low which will affect the treatment process. If any fluctuation occurs in the production process and the wastewater entering the treatment system with high organic load than at that time the entire system fails because this will not be encountered properly by the low MLSS treatment system. The three levels selected for F/M ratio were 0.5, 1.0 and 1.5.

The fourth parameter selected was Dissolved Oxygen (DO). DO is required by the microorganisms to respire properly. Too much oxygen adds unnecessary cost due to increased power consumption and too little can decrease the metabolism of the micro-organisms and the efficiency of the process. The optimum range for the DO in the treatment plants were in between 0.5 - 1.5 mg/l. The levels selected for our study in case of DO were 0.5, 1.0 and 1.5 mg/l.

The fifth parameter selected was hydraulic react time. Waste water aeration time, usually expressed in hours, is based on the time required by the microorganisms to degrade the organic load present therein. Hydraulic react time is selected on the basis of the characteristics and strength of wastewater and the desired degree of treatment. It is an important parameter because industrial persons cannot hold their

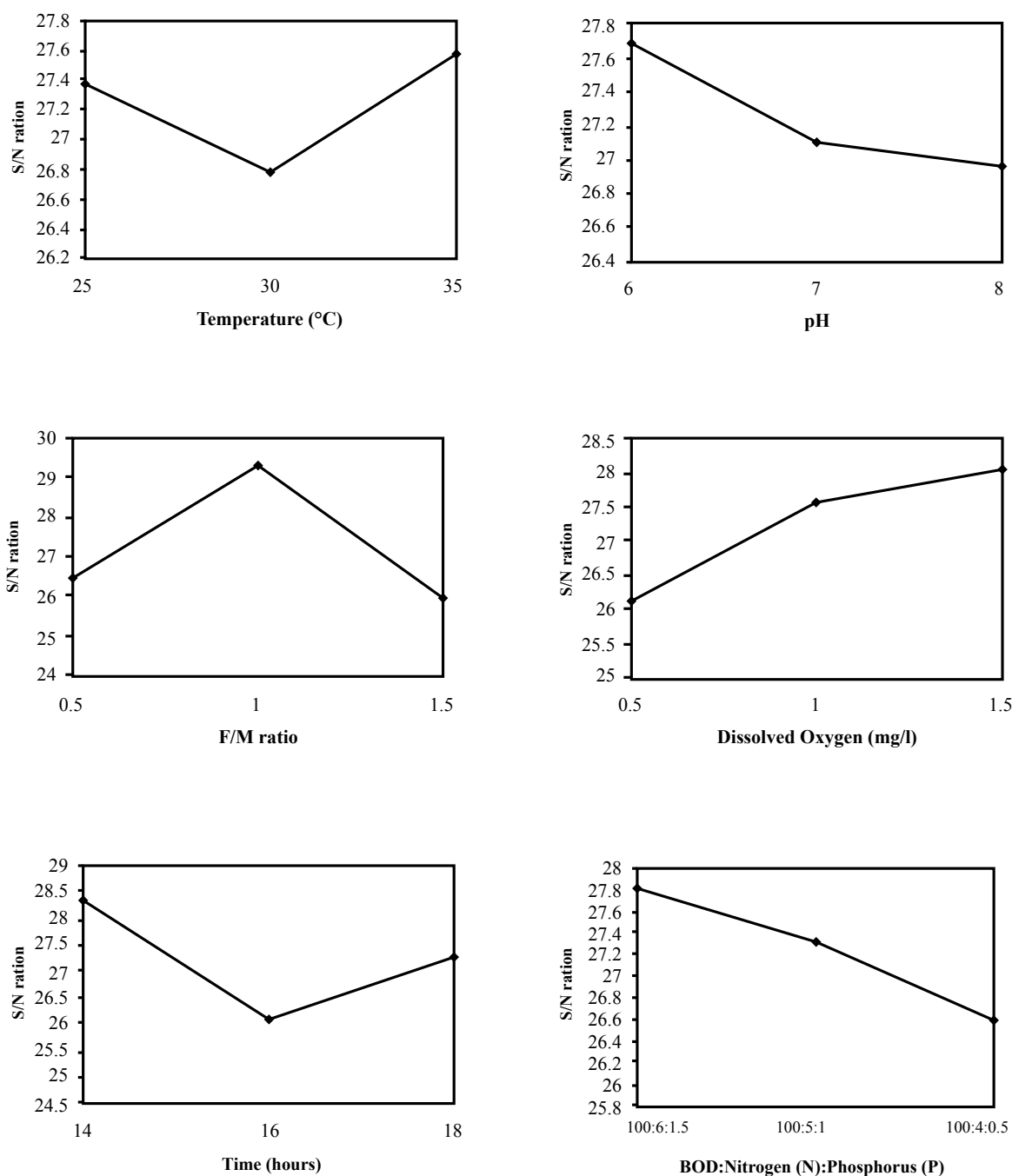


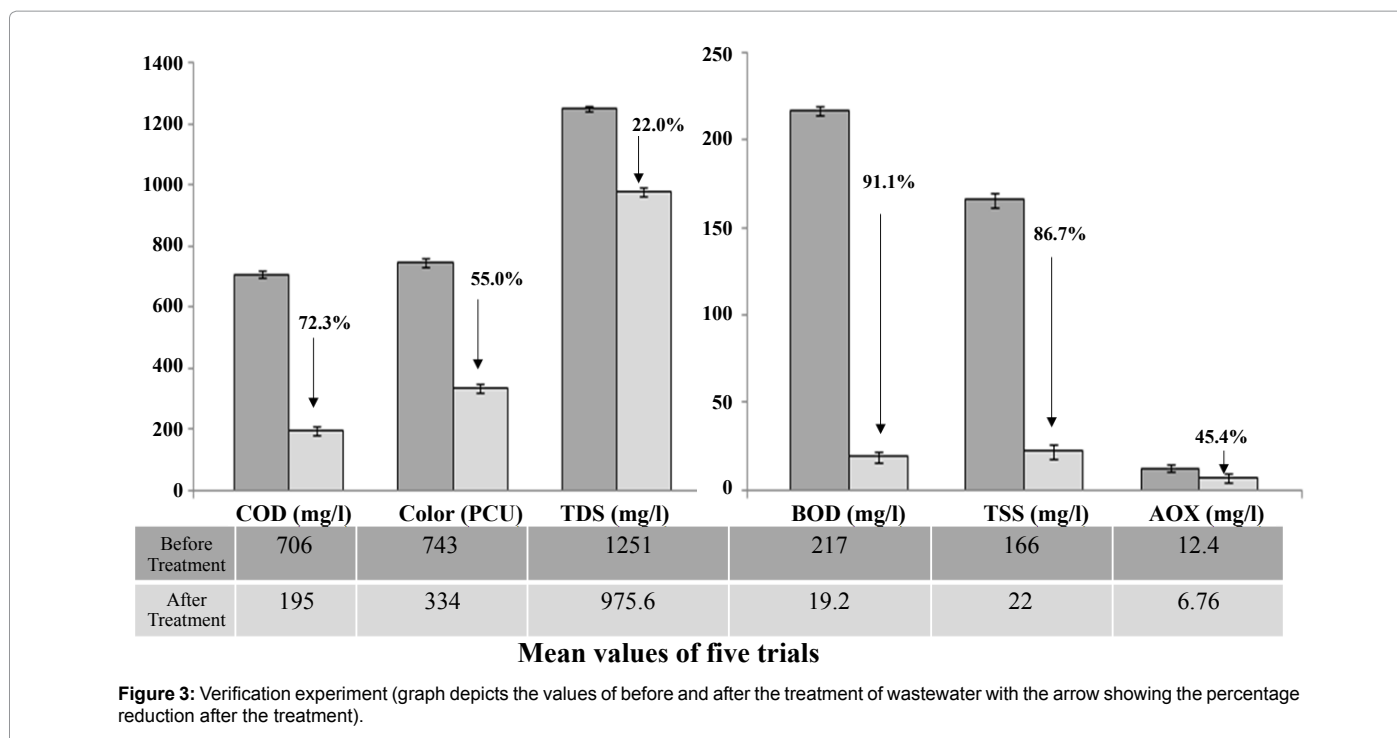
Figure 2: Level average response graphs by S/N ratio: (a) temperature (b) pH (c) F/M ratio (d) dissolved oxygen (e) incubation time (f) BOD:N:P (Nutrient supplements).

wastewater within their premises for longer period. Therefore in our study the three levels selected for treatment time were 14hrs, 16 hrs and 18 hrs.

Last parameter selected was nutrient dosing. Nitrogen and Phosphorus are the essential elements other than the carbon for the proper growth and activity of the microorganism present in the aeration chamber [28]. Pulp and paper industrial effluent is rich in carbon source due the raw material used but, wastewater is deficient in N and P [29]. For effective treatment of wastewater, significant quantity of N

and P must be added because microorganisms present in the effluent require N and P to produce enzymes for the degradation of organic matter present. Insufficient N can result in filamentous and dispersed growth of microbial population which settles poorly. As a general rule the ratio of N & P required with respect to BOD load is 100 BOD :5 N :1 P. Keeping in mind the standard values, three levels selected for the nutrient dosing were 100:6:1.5, 100:5:1 and 100:4:0.5.

Verification experiment: The levels selected by Taguchi approach for various parameters were tested in order to verify the results of



optimum conditions. The experiments were run five times to check the error. Mean values of the COD, color, TDS, BOD, TSS and AOX before and after treatment of the wastewater were plotted in the Figure 3. After observing the results the reduction obtained in case of COD, color, TDS, BOD, TSS and AOX were 72.3%, 55%, 22%, 91%, 86.7% and 45.4%, respectively with 52 Sludge Volume Index (SVI). Sludge volume index is important parameter for the treatment of wastewater. SVI is used to assess the settling qualities of sludge. The volume of settled sludge is read after every trial. The minimum and maximum values were 65 and 40 respectively. It is reported in literature that SVI can vary from 30 - 400 ml g⁻¹ [30-32]. But it is also mentioned that if the value increased from 150 ml g⁻¹ the plant operator should face the problem of sludge bulking [33].

Environment friendly manner for reducing COD, Colour, BOD and AOX of pulp and paper effluent is in demand. The method should not be much time consuming and less expensive. Several physico-chemical methods were used they are efficient but more expensive. Recently researchers were concentrated on bacterial and fungal degradation of the pulp and paper effluent in a less expensive and eco-friendly manner. The use of white rot and soft rot fungal species for treatment of the effluent by Freitas et al. [34] results in 74-81% of COD reduction after 10 days, while comparing the results with this study it was observed that to complete the process of COD & BOD reduction the time required was 16-18hr as compare to 10 days [34]. Some researchers explored purified fungal cellobiose dehydrogenate for color remediation of pulp mill effluent and could remove up to 50% color after 4 days on the otherhand the color reduction was upto 55% in our case and the time taken to complete the process was 16-18hrs. [35]. But, fungal treatment of pulp and paper mills effluent was not feasible because fungi are unable to proliferate under extreme environmental conditions (high pH, temperature, and oxygen limitation) that are present in effluent treatment plant of agro-based pulp and paper mills. In addition, fungal filaments cause structural hindrance in effluent treatment plants. Researchers were also using bacteria for the treatment

of pulp and paper industrial wastewater. The strains *Bacillus cereus* (ITRC - S6) and *Serratia marcescens* (ITRC-S7) were used by Chandra et al. [36] and results showed that the strains were effectively reduced colour (45-52%), lignin (30-42%), BOD (40-70%), COD (50- 60%), in a 7-day period [36]. The complete process will take 7 days to reduce the wastewater parameter color, lignin BOD and COD whereas in our study the time taken to reduce the parameters were 16-18hrs and reduction was also more. In another study Raj et al. [19] demonstrated that *Bacillus sp.* was able to remove 61%, 53%, 82% and 78% of colour, lignin, BOD and COD within 6 days of incubation [37]. Another researcher Gupta et al. [37] reported that the two strains were able to remove 70% to 80% of COD, and lignin's while the colour around 85% in 8 days of detention time [37]. Three bacterial isolates used by Chandra et al. [38] reported the degradation of Kraft lignin which is able to reduce 69% colour, 40% lignin and total substrate by 50% after incubating the sample for 48 h [38]. The use of sulphate reducing bacteria by Hao and Man [39] able to remove COD up to 70-75% after 3 weeks and increase to 82-88% by subsequent aerobic treatment for 48 h [39]. Fungus and bacteria in combination were also used by some researchers to increase the degradation rate. Chupal et al. [40]; use fungus followed by bacteria for the treatment of wastewater [40]. The fungal-treated wastewater was again treated with the bacteria for the biodegradation process used *Paecilomyces sp.* and *Pseudomonas syringae pv. myricae* and reported significant reduction in colour (88.5%), lignin (79.5%), COD (87.2%) and phenol (87.7%) in two steps. Ghoreishi and Haghghi [41] used chemical and biological reactions in series and reported 99% of BOD and 92% of COD and 97% of TSS reduced after 6 days of incubation [41]. After comparing the results with previous studies the time taken to complete the degradation process was less in our case. In less incubation time more degradation was achieved in eco-friendly manner.

Identification: Identification of bacterial isolates of consortium was carried out by 16S rRNS gene sequencing studies. Partial 16S rRNA sequence data of the isolates PNP3, PNP6 and PNP8 were analyzed by BLAST using the programme available online at National Centre for

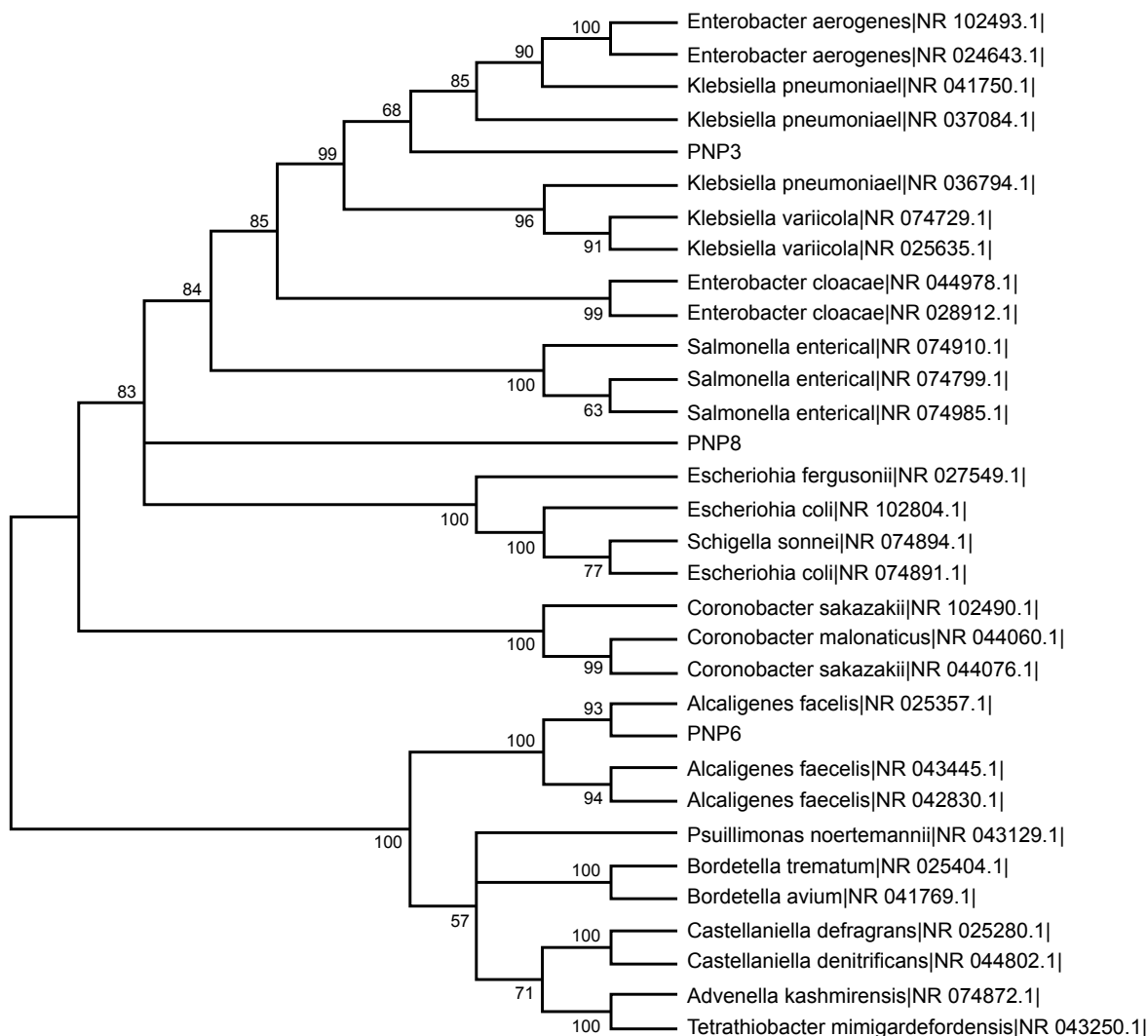


Figure 4: Neighbor-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationship between strain PNP3, PNP6, PNP8 and their related genera (sequence was downloaded from GenBank). Their names and respective accession numbers are given in the tree. Bootstrap Values (expressed as percentage of 1000 replications) of more than 60 are shown at branch points. Space bar represents 0.02 substitutions per site.

Biotechnology Information (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). BLAST result analysis of lab isolate PNP3, PNP6 and PNP8 showed identity with *Klebsiella sp.* (99%, accession no NR_074913.1), *Alcaligenes sp.* (99%, accession No. NR_025357.1) and *Cronobacter sp.* (97%, accession No. NR_102490.1), respectively.

The 16S rRNA gene sequence of PNP3, PNP6 and PNP8 has been submitted to GenBank under accession numbers KF531636, KF531637 and KF531638 respectively. Phylogenetic analysis was carried out using these strains reported in Gen- Bank and the results are shown in Figure 4.

Survival of bacteria in SBR: The current study also emphasized the survivable of bacterial isolate of selected consortia in the effluent after successful compilation of the bioremediation study. Results showing the presence of microbial consortia in the sludge sample. The developed microbial consortium consists of bacterial isolates namely *Klebsiella sp.*, *Alcaligenes sp.* & *Cronobacter sp.* It has been observed from the results that out of the total microbial flora 80% was the developed

consortium in the SBR. The 90 colony forming unit (CFU) was present on the plate out of which 72 CFU were the mixture of the developed consortium. Rest of 18 was another micro flora present in the sludge itself. This experiment was repeated thrice in order to check the error among the results. Survival of bacterial consortium in the effluent during the treatment process played an important role in the reduction of pollution.

Conclusion

Bacteria were isolated from the soil samples, collected from the premises of pulp and paper industry having lignocellulosic compound degrading capability and having the potential to degrade pollutants present in pulp and paper industrial effluent. It was observed that bacteria when used in group as consortium showed better results in reducing the wastewater parameters as compared to single isolates. Selected consortium can effectively treat the wastewater holistically. After 16 - 18 hrs of incubation results showed the achieved reduction in COD, color, TDS, BOD, TSS and AOX were 72.3%, 55%, 22%, 91%,

86.7% and 45.4% respectively with 52 sludge volume index (SVI). At F/M ratio - 1.0 and SVI 52 the sludge showed better settleability. The results obtained were within the permissible limit for the discharge provided by regulatory agency. The strains were identified by using 16S rRNA gene sequence analysis technique. The strains were identified as *Klebsiella sp.* (99%, accession no NR_074913.1), *Alcaligenes sp.* (99%, accession No. NR_025357.1) and *Cronobacter sp.* (97%, accession No. NR_102490.1). The results of survivability showed that the bacteria were not only effective but also dominant irrespective of the other strains present in the effluent. After observing and comparing our study with the previous one we can say that it can be effectively applicable to the field scale because the conditions were very much comparable to the field conditions.

Acknowledgement

The authors acknowledge the financial help and infrastructure provided by the CSIR-IGIB. The authors also acknowledge the industrial persons for the cooperation for providing wastewater samples, whenever required and the generous hospitality offered to us upon each visit.

References

1. Ali M, Sreerishnan T (2001) Aquatic toxicity from pulp and paper mill effluents: a review. J Adv Environ Res 5: 175–196.
2. Yadav BR, Garg A (2011) Treatment of pulp and paper mill effluent using physico-chemical processes. IPPTA J 23: 155-160.
3. Chaudhary S, Rohella R, Manthan M, Sahoo N (2002) Decolorization of Kraft paper mill effluent by white rot fungi. J Microbiol 38: 221-224.
4. Hossain MSK, Das M, Ibrahim SH (2001) Aerobic studies on pollution abatement of sulfite pulp bleaching effluent using *Phanerochaete chrysosporium* (MTCC-787). J Ind Poll Con 17: 191-200.
5. Jayaramraja PR, Anthony T, Rajendran R, Rajkumar K (2001) Decolorisation of paper mill effluent by *Aspergillus fumigatus* in bioreactor. Poll Res 20: 309-312.
6. Pokhrel D, Viraraghavan T (2004) Treatment of pulp and paper mill wastewater - a review. Sci Total Environ 333: 37-58.
7. Sundman G, Kirk TK, Chang HM (1981) Fungal decolorization of kraft bleach effluent: fate of the chromophoric material. TAPPI 64: 145-148.
8. Crawford RJ, Styker MN, Jett SW, Carpenter WL, Fisher RP, et al. (1987) Lignin degradation and transformation. J Tappi 70: 123.
9. Savant DV, Abdul-Rahman R, Ranade DR (2006) Anaerobic degradation of adsorbable organic halides (AOX) from pulp and paper industry wastewater. Bioreour Technol 97: 1092-1104.
10. Chang CN, Ma YS, Fang GC, Chao AC, Tsai MC, et al. (2004) Decolorizing of lignin wastewater using the photochemical UV/TiO₂ process. Chemosphere 56: 1011-1017.
11. Yeber MC, Oñate KP, Vidal G (2007) Decolorization of kraft bleaching effluent by advanced oxidation processes using copper (II) as electron acceptor. Environ Sci Technol 41: 2510-2514.
12. Perez M, Torrades F, Gracia-Hortal JA, Domenech X, Peral J (2002) Removal of organic contaminants in paper pulp treatment effluents under Fenton and photo-Fenton conditions. Appl Catal 36: 63–74.
13. Kazmi AA, Thul R (2007) Colour and COD removal from pulp and paper mill effluent by q Fenton's oxidation. J Environ Sci Eng 49: 189-194.
14. Zahrim AY, Gilbert ML, Janaun J (2007) Treatment of pulp and paper mill effluent using photo-Fenton's process. J Appl Sci 7: 2164–2167.
15. Ganjidoust H, Tatsumi K, Yamagishi T, Gholian RN (1997) Effect of synthetic and natural coagulant on lignin removal from pulp and paper wastewater. Water Sci Technol 35: 291–296.
16. Singh P, Singh A (2004) Physico-chemical characteristics of the distillery effluent and its chemical treatment. J Nature Sci Technol 3: 205–208.
17. Barton DA, Lee JW, Buckley DB, Jett SW (1996) Biotreatment of kraft mill condensates for reuse. Proc Tappi Minimum Effluent Mills Symp, GA Atlanta, USA.
18. Nagarathamma R, Bajpai P, Bajpai PK (1999) Studies on decolorization, degradation and detoxification of chlorinated lignin compounds in kraft bleaching effluents by *Ceriporiopsis subvermispora*. Process Biochem 34: 939–948.
19. Raj A, Reddy MM, Chandra R, Purohit HJ, Kapley A (2007) Biodegradation of kraft-lignin by *Bacillus sp.* isolated from sludge of pulp and paper mill. Biodegradation 18: 783-792.
20. Lenore S. Clesceri (1998) Standard Methods for the Examination of Water and Wastewater, American Public Health Association/American Water Works Association/Water Environment Federation, Washington DC, USA.
21. NCASI (1999) Method for analysis of color of pulp and paper industrial waste water. National Council for Air and Stream Improvement Inc. USA.
22. Thakur IS, Verma PK, Upadhaya KC (2001) Involvement of plasmid in degradation of pentachlorophenol by *Pseudomonas sp.* from a chemostat. Biochem Biophys Res Commun 286: 109-113.
23. Lane DJ (1991) 16S/23S rRNA sequencing. In: Nucleic acid techniques in bacterial systematics. Stackebrandt, E., and Goodfellow, M., eds., John Wiley and Sons, New York, USA.
24. Selina Bamforth, Ian Singleton (2005) Bioremediation of polycyclic aromatic hydrocarbons: current knowledge and future directions. J Chem. Technol Biotechnol. 80:723-736.
25. Brahmani PSR, Mugeraya G, Sandhyavali MS (2012) Effect of media constituents on microbial enzyme activity. Inter J Phrma Chem Biolo Sci. 2: 236-241.
26. Kumar V, Dhall P, Kumar R, Prakash Singh Y, Kumar A (2012) Bioremediation of agro-based pulp mill effluent by microbial consortium comprising autochthonous bacteria. ScientificWorldJournal 2012: 127014.
27. Michael HG (2002) Nitrification and Denitrification in the Activated Sludge Process. Copyright John Wiley & Sons, Inc, USA.
28. U.S. Environmental Protection Agency (USEPA) (2012). In-Situ Groundwater Bioremediation, USA.
29. Dijkstra P, Thomas SC, Heinrich P L, Koch GW, Schwartz E, et al. (2011) A Effect of temperature on metabolic activity of intact microbial communities: Evidence for altered metabolic pathway activity but not for increased maintenance respiration and reduced carbon use efficiency. Soil Biol Biochem 43: 2023–2031.
30. Janczukowicz W, Szewczyk M, Krzemieniewski M, Pesta J (2001) Settling Properties of Activated Sludge from a Sequencing Batch Reactor (SBR). Polish J Environ Stud 10: 15-20.
31. Hanel K (1988) Biological Treatment of Sewage by the Activated Sludge Process. Ellis Horwood Limited, New-York - Chichester - Brisbane - Toronto.
32. Ganczarzyk J (1969) Waste water Treatment by the Activated Sludge. Arkady, Warsaw.
33. Palm J, Jenkins D (1980) Relationship Between Organic Loading, Dissolved Oxygen Concentration and Sludge Settleability in the Completely Mixed Activated Sludge Process. Journal WPCF 10: 2484.
34. Freitas AC, Ferreira F, Costa AM, Pereira R, Antunes SC, et al. (2009) Biological treatment of the effluent from a bleached kraft pulp mill using basidiomycete and zygomycete fungi. Scien Total Environ 407: 3282–3289.
35. Wingate KG, Stuthridge T, Mansfield SD (2005) Colour remediation of pulp mill effluent using purified fungal cellobiose dehydrogenase: reaction optimisation and mechanism of degradation. Biotechnol Bioeng 90: 95-106.
36. Chandra R, Raj A, Yadav S, Patel DK (2009) Reduction of pollutants in pulp paper mill effluent treated by PCP-degrading bacterial strains. Environ Monit Assess 155: 1-11.
37. Gupta VK, Minocha AK, Jain N (2001) Batch and Continuous Studies on Treatment of Pulp Mill Wastewater by Bacterial Strain *Aeromonas formicans*. J Chem Tech Biotech 76: 547–552.
38. Chandra R, Raj A, Purohit HJ, Kapley A (2007) Characterisation and optimisation of three potential aerobic bacterial strains for kraft lignin degradation from pulp paper waste. Chemosphere 67: 839-846.
39. Hao DT, Man TD (2006) Study on treatment of alkaline black liquor using sulphate reducing bacteria. Adv Natural Sci 7: 139–144.
40. Chuphal Y, Kumar V, Thakur IS (2005) Biodegradation and decolorization of pulp and paper mill effluent by anaerobic and aerobic microorganisms in a sequential bioreactor. World J Micro Biotech 21: 1439–1445.
41. Ghoreishi SM, Haghghi MR (2007) Chromophores removal in pulp and paper mill effluent via hydrogenation biological batch reactors. Chem Engin J 127: 59–70.