

## USE OF *BACILLUS PUMILUS* CBMAI 0008 AND *PAENIBACILLUS* SP. CBMAI 868 FOR COLOUR REMOVAL FROM PAPER MILL EFFLUENT

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### ABSTRACT

*Bacillus pumilus* and *Paenibacillus* sp. were applied on the paper mill effluent to investigate the colour remotion. Inocula were individually applied in effluent at pH 7.0, 9.0 and 11.0. The real colour and COD remotion after 48h at pH 9.0 were, respectively, 41.87% and 22.08% for *B. pumilus* treatment and 42.30% and 22.89% for *Paenibacillus* sp. Gel permeation chromatography was used to verify the molar masses of compounds in the non-treated and treated effluent, showing a decrease in the compounds responsible for the paper mill effluent colour.

**Key words:** *Bacillus pumilus*; *Paenibacillus* sp.; colour removal; paper mill effluent.

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### INTRODUCTION

The paper industries clearly have to face more severe legal restrictions concerning environmental pollution. The new laws have been forced the paper companies to implement relevant changes in their manufacturing processes (7).

One of the specific problems that have not been solved until now is the strong dark colour of the effluents, which is primarily due to lignin and its derivatives released from the substrate and discharged in the effluents, mainly from the pulping, bleaching and chemical recovery stages (11). Dark colour of the effluent is not only aesthetically unacceptable but can increase water temperature and decrease the photosynthesis; both can lead to decreased concentration of dissolved oxygen (5). Lignin and its derivatives are difficult to degrade because of the molecule linkages, especially the biphenyl type carbon-to-carbon linkages.

Bacterial aerobic and anaerobic treatment systems can reduce the biological oxygen demand (BOD) and are able to remove the dark colour in the effluents as verified previously (14,15). However, even though some physical and chemical methods quite effective in decolorization of pulp and paper mill

effluents present severe setbacks such as high cost per unit volume of wastewater treated or unreliability in operation (4,17).

Several researches have shown that a group of extracellular isoenzymes, known as ligninases, which are lignin peroxidase (LiP), manganese-dependent peroxidase (MnP) and laccase, produced by some microorganisms are capable of degrading lignin present in the paper mill effluent. This treatment can be a more cost effective alternative. *Bacillus pumilus* CBMAI 0008 and *Paenibacillus* sp. CBMAI 868 previously isolated, respectively, from wood decomposition material and paper mill effluent were identified by the Coleção Brasileira de Microorganismos de Ambiente e Indústria - CBMAI and are able to produce alkaline enzymes under thermophilic conditions, including xylanases and manganese-dependent peroxidase (1,2,3,8,10). In this study we evaluated the ability of these bacteria for colour removal of the paper mill effluent.

### MATERIALS AND METHODS

#### Effluent source

Effluent from first extraction of the bleaching sequence was collected from a paper industry, located in Americana - SP,

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Brazil. For the study, samples from acidic (pH = 3.0) and alkaline (pH = 12.0) treatments were collected and mixed to reach the desirable pH (pH 7.0, pH 9.0 and pH 11). Samples were filtered to remove large suspended particles and stored at -25°C until use.

### Microorganisms.

*Bacillus pumilus* CBMAI 0008 was isolated from wood decomposition material by Duarte *et al.* (1) and was maintained in a culture medium containing birchwood xylan (6). *Paenibacillus* sp. CBMAI 868 was isolated from paper mill wastewater, in a previously study (10) in a medium containing (%): birchwood xylan (Sigma), 1; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1; paper mill wastewater, 50; agar-agar, 2. The medium was sterilized at 121°C for 15 min, and nistatin (44.0 mg.L<sup>-1</sup>) was added as an antifungal control. Both bacteria were isolated at 45°C and are able to growth and produce enzymes in this condition.

### Inocula preparation

The cultures were individually transferred to 125 mL Erlenmeyer flasks, containing 12.5 mL of the liquid medium (6), and incubated at 45°C in a rotary shaker (250 rpm) for 20 h. Indulin AT (0,1% w/v) was added as inducer in the medium from *Paenibacillus* sp., once this stimulate manganese-dependent peroxidase production by this bacterium as demonstrated in a previous study (10). After growth cell concentrations of the two different cultures were adjusted to 3% T (transmittance) in relation to distilled water at 600 nm.

### Decolorization studies

*B. pumilus* and *Paenibacillus* sp. were investigated individually in the crude paper mill effluent at pH values adjusted as described previously. Decolorization tests were conducted in 250 mL Erlenmeyer flasks, containing 100 mL of effluent. After bacteria inoculation (8% v/v, equivalent to 10<sup>8</sup>/mL), the flasks were incubated under 200 rpm at 45°C during 24 h or 48 h, and were centrifuged at 10.000 rpm under refrigeration.

### Real colour measurement

Real colour was measured according to Standard Methods (16). Colour was determined in a HACH - model DR2010 spectrophotometer at 455 nm. Colour quantification was done through directly reading of untreated and treated effluent samples. Results were expressed in mg Pt-Co/L.

### Chemical oxygen demand measurement

Chemical oxygen demand (COD) was determined in untreated and treated effluent by a closed reflux colorimetric method (method 5220 C) according to Standard Methods (16). A HACH - model DRB 200A was used for digestion of sample in COD vials. COD was spectrophotometrically determined at 600 nm.

### Gel permeation chromatography

The molecular weight distribution in untreated and treated effluent (best colour remotion and COD results) was determined by loading 2 mL of each sample (centrifuged effluent samples supernatant) onto a Sephadex G-75 resin (KX-16 100/16). The resin was previously equilibrated with NaOH (0.002 g/L) and LiCl (0.848 g/L) in isocratic system, flow at 2.0 mL/min, connected to a GradiFrac (Pharmacia Biotech) (12). Sample elution was monitored by OD<sub>280nm</sub> in a Pharmacia LKB UV-1 detector.

## RESULTS AND DISCUSSION

### Effluent Decolorization

As reported for fungi in previous studies, the decolorization tests of the paper mill effluent was carried out using whole cell of each bacteria. The real colour of the untreated effluent at pH 7.0, 9.0 and 11.0 were, respectively, 887, 781 and 597 mg Pt-Co/L. The percentage of real colour reduction after 24 h and 48 h using *B. pumilus* and *Paenibacillus* sp. treatments are shown in Table 1. According to results, the treatment using *Paenibacillus* sp., in general, was more efficient than *B. pumilus* treatment, and was more pronounced at pH 7.0. In this condition, *Paenibacillus* sp. reduced the effluent colour in 41.08% after 48 h, while *B. pumilus* allowed 24.01% of colour reduction. At pH 9.0 the action of the microorganisms was similar, being observed 42.30% of colour reduction for *Paenibacillus* sp. and 41.87% for *B. pumilus*.

The results from Table 1 also show that *Paenibacillus* sp. was efficient in a larger pH range than *B. pumilus* (pH 7.0 to pH 9.0). These results suggest that the enzymatic system from two microorganisms can be different or the bacteria produce enzymes whose optimal activity occurs in different pH values. However, further assays must be conducted in order to determine this question.

**Table 1.** Effluent real colour reduction in different pH values after treatment with *B. pumilus* and *Paenibacillus* sp.

Effluent (pH)	Treatment Time (h)	Colour reduction after treatment			
		<i>B. pumilus</i>		<i>Paenibacillus</i> sp.	
		Value	%	Value	%
		Mg Pt-Co/L		Mg Pt-Co/L	
7.0	24	182.99 ± 2.62	20.63	204.36 ± 3.77	23.04
	48	212.97 ± 2.72	24.01	364.38 ± 1.74	41.08
9.0	24	228.36 ± 2.34	29.24	283.66 ± 4.72	36.32
	48	327.00 ± 0.78	41.87	330.36 ± 5.78	42.30
11.0	24	169.67 ± 1.25	28.42	241.02 ± 4.73	35.85
	48	178.98 ± 1.41	29.98	188.35 ± 1.23	31.55

Recent studies using ligninolytic enzymes from filamentous fungi as *Aspergillus fumigatus* and *A. flavus* showed a removal from the paper effluent colour (12). In this case, *A. fumigatus* was able to decolorize 55.5% under stationary condition and 89.3% under shaking conditions, while *A. flavus* removed 53.5 and 84.0%, respectively, in the same conditions. The difference in the colour reduction was related to differences in aeration between stationary and shaking flasks conditions. Basidiomycetes were also applied in the paper effluent aim decoloration, as *Pleurotus sajor-caju*, *P. platypus* and *P. citrinopileatus* (11). After 6 days, *P. sajor-caju* decolorized 66.7% of the effluent. These studies show a decolorization ranges between 55% to around 90%. However, in order to obtain such efficiency, is necessary 3 to 6 days of treatment, which can not be applied industrially.

The use of bacterial enzymes for effluent colour removal in the paper industry was reported previously (4,14,15).

In the present work, was obtained about 40% efficiency in the colour decolorization after 48 h. Further investigations should be conducted to show if an increase in the treatment time of the effluent with these bacteria, can allow the same efficiency reached for fungi.

#### Chemical oxygen demand measurement (COD)

Untreated and treated effluent by *B. pumilus* and *Paenibacillus* sp. were evaluated to COD. The media values for COD in the untreated effluent were 2157.92, 2221.68 and 2297.59 mg/mL, when the pH was corrected to pH 7.0, 9.0 and 11.0, respectively. The percentage of COD reduction after 24 h and 48 h from *B. pumilus* and *Paenibacillus* sp. treatment are shown in Table 2. The results show that although not occurred important changes after 24 h or 48 h, was observed a little increase in the COD reduction when the pH was raised from pH 7.0 to pH 11.0. Considering the efficiency in the COD reduction, about 22% at pH 9.0 was observed for both bacteria, around

**Table 2.** Effluent COD reduction in different pH values after treatment with *B. pumilus* and *Paenibacillus* sp.

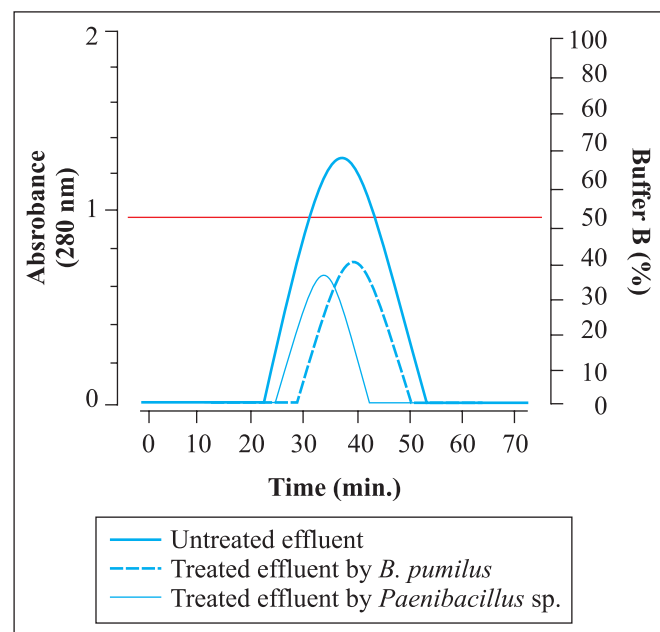
Effluent (pH)	Treatment Time (h)	Colour reduction after treatment			
		<i>B. pumilus</i>		<i>Paenibacillus</i> sp.	
		Value mg/mL	%	Value mg/mL	%
7.0	24	441.51 ± 2.00	20.46	412.38 ± 2.82	19.11
	48	457.26 ± 8.35	21.19	418.85 ± 3.02	19.41
9.0	24	487.66 ± 1.99	21.95	496.32 ± 2.26	22.34
	48	490.55 ± 1.15	22.08	508.54 ± 4.50	22.89
11.0	24	531.43 ± 2.94	23.13	544.52 ± 3.89	23.70
	48	541.77 ± 3.38	23.58	549.35 ± 2.90	23.91

half from that reported for the fungus *Ceriporiopsis subvermispora*. In a previous study, this microorganism was able to remove 45% of COD of the paper mill effluent also after 48 h (9). Another fungus, *Pleurotus sajor-caju*, could remove 61.3% of COD after 10 days (11), while strains of *Fomes lividus* and *Trametes versicolor* reduced paper mill effluent COD in 66.7% and 59.7%, respectively, after 10 days (13). The removal observed employing fungi is very superior to those found in this work using *B. pumilus* and *Paenibacillus* sp.

#### Gel permeation chromatography

The molecular weight distribution of the compounds present in the untreated and treated paper effluent was determined by gel permeation chromatography. Samples of the treated effluent that showed best results in real colour remotion were selected for evaluation. The molecular weight distribution in the untreated and treated effluent for 48 h at pH 9.0 is presented in Fig. 1.

The determination of the molecular size distribution in decolorized effluent is useful for determining the modification that occurs in chromotrophic groups responsible for colour (12). Through comparison of the eluted peaks from crude effluent and treated effluent with microorganisms (Fig. 1) was possible to notice a decrease in the molecular weight in this last. The peaks areas were reduced nearly 60% and 70% with *B. pumilus*



**Figure 1.** Sephadex G-75 ellution pattern at 280 nm obtained with untreated effluent and treated effluent with *B. pumilus* and *Paenibacillus* sp. at pH 9.0. NaOH and LiCl buffer in isocratic system, flow at 2.0 mL/min.

and *Paenibacillus* sp., respectively, confirming that the compounds present in the paper effluent were depolymerized during treatment. These changes were also noticed in paper mill effluent treated with *A. fumigatus* (12). There was a significant decrease in high- and medium-molecular weight compounds and presence of small amount of low-molecular weight compounds in decolorized effluent, confirming the depolymerization and biochemical degradation of high-molecular weight coloured compounds by *A. fumigatus*. *Ceriporiopsis subvermisporea* was also able to degrade compounds present in the paper mill effluent (9). FPLC/GPC chromatogram indicated that the macromolecules present in effluent had molecular weights of 16000 Da. After treatment with *C. subvermisporea* macromolecules were degraded in low molecular weight compounds (< 1 kDa).

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#### RESUMO

##### **Emprego de *Bacillus pumilus* CBMAI 0008 e *Paenibacillus* sp. CBMAI 868 para remoção da cor do efluente da indústria papelreira**

*Bacillus pumilus* e *Paenibacillus* sp. foram aplicados separadamente no efluente da indústria papelreira a pH 7,0, 9,0 e 11,0, para verificação da remoção da cor e da DQO. As remoções da cor real e DQO após 48h a pH 9,0 foram, respectivamente, de 41,87% e 22,08% após o tratamento com *B. pumilus* e 42,30% e 22,89% após tratamento com *Paenibacillus* sp. As massas molares dos compostos presentes no efluente não tratado e tratado foram determinadas por cromatografia de permeação em gel. O emprego dos microrganismos reduziu os compostos responsáveis pela cor do efluente da indústria papelreira.

**Palavras-chave:** *Bacillus pumilus*; *Paenibacillus* sp.; remoção da cor; efluente da indústria papelreira.

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