Characterization of Culturable Bacteria from Pulp and Paper Industry Wastewater, with the Potential for Degradation of Cellulose, Starch, and Lipids

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The search for microbial enzymatic activities applied to wastewater treatment is an important task in environmental biotechnology. Microbial enzymes have been previously explored in hostile habitats. They are increasingly important in extreme habitats; biological wastewater from the pulp and paper mill industry can harbor microorganisms with valuable enzymatic capabilities that can improve the efficiency for the same process of depuration. This study was performed to characterize and evaluate cellulolytic, amylolytic, and lipolytic activities of bacteria isolated from a pulp and paper effluent. The enzymatic activities were evaluated by the formation of a clear halo around the colonies in defined substrate media. By the use of a sequence analysis of 16S rDNA libraries, isolates were identified. The 16S rDNA libraries belong to the Bacillus subtilis, B. megaterium, B. licheniformis, B. pumilus, B. thuringiensis, B. cereus, Chryseobacterium daecheongense, and Microbacterium sediminis (an alkali-tolerant bacteria which has only been isolated from deep-sea sediment). B. cereus was the best strain for cellulose and lipase activities; moreover, C. daecheongense was best for amylase activity. The present study shows that the aerated lagoons from the pulp and paper industry are a promising source of bacterial with different enzyme activities. This data is relevant for industrial applications.

Keywords: Pulp and paper effluent; Enzymatic activity; DNA libraries; Isolated-bacteria

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INTRODUCTION

A wastewater treatment plant is designed to remove most of the organic matter in wastewater through microbial processing with important enzymatic activities (Molina-Muñoz et al. 2010). The search for microbial enzymatic activities applied to wastewater treatment is an important task in environmental biotechnology. Specific contaminants can be eliminated or transformed by the action of enzymes (Karam and Nicell 1997). Martínez-Martínez et al. (2017) mention that by 2030 approximately 40% of chemical synthesis processes will be replaced by enzymatic processes. There are many factors that affect the enzymatic treatment efficiency such as pH and limited stability to extremes of temperature (Bajpai 1999; Hough and Danson 1999). Depending on the isolation habitat, a variety of bacteria produce different enzymes. Microbial enzymes have been explored previously in...
hostile habitats, due to their potential use under these conditions (Littlechild 2015). The enzymatic activities enable the bacteria to adapt to extreme conditions, to biodegrade various compounds, to produce several metabolites, and to detoxify metal compounds (Kulshreshtha et al. 2010).

The undiscovered microbial diversity is a treasure for biotechnological applications (Streit and Schmitz 2004; Liu and Kokare 2017). However, more than 99% of microbial species are “uncultured”, because the environmental conditions of growth are unknown or different (Rondon et al. 1999; Hugenholtz 2002). In silico analysis of uncultured bacterial have provided much information about specific genes; however, the function of a gene cannot be determined without experimental testing (Srivastava et al. 2016).

The effluent from pulp and paper industry mills is an extreme and polluted environment. It can harbor microorganisms with valuable enzymatic capabilities capable of improving the efficiency of the same process of depuration (Karrasch et al. 2006). Pulp and paper industry processes offer numerous opportunities for the application of microbial enzymes (Bajpai 1999).

Wastewater from pulp and paper making processes are an important source of cellulose and contain soluble components such as starch (Thompson et al. 2001). Moreover, activated sludge contains cellulose (2 to 8%) and lipids (2 to 10%) (Kyllönen et al. 1988). For the degradation of these compounds, fungi are effective in cellulose hydrolysis (Persson et al. 1991; Hansen et al. 2015; Ordaz-Díaz et al. 2016). Nevertheless, of the microorganisms that participate in wastewater depuration, bacteria are the main contributor (Forster et al. 2003). Bacteria with the ability to hydrolyze proteins, starch, and lipids, have been used in the past for the treatment of wastewater (Gratia et al. 2009). However, few studies have been carried out on the isolation and evaluation of the enzymatic activities of native bacteria from pulp and paper mill effluents.

An option for biological wastewater treatment is inoculation or bioaugmentation with bacteria (previously isolated) that present high enzymatic activity and have been previously adapted to extreme environmental conditions. This research was conducted to explore culturable bacteria and to identify and evaluate the cellulolytic, lipolytic, and amylolytic activities of strains isolated from a pulp and paper mill effluent. The goal was to reveal potential bacteria for the bioremediation of wastewater from pulp and paper mill industrial sites.

EXPERIMENTAL

Materials
Water samples were collected from an effluent lagoon of a pulp and paper mill. The samples were collected and transported in sterile glass jars and coolers for conservation. For bacteria isolation, serial dilutions of water samples were prepared. 100 μL of the wastewater sample was spread on Luria-Bertani (LB) agar plates and were incubated at 37 °C for 24 h. LB agar-plates contained 10 g peptone (Merck, Darmstadt, Germany), 5 g sodium chloride (Baker Analyzed®, Edo. de Mex., Mexico), 5 g yeast extract (Bexton Dickinson Bioxon, Edo. de Mex., Mexico), and 23 g agar in 1000 mL distilled water, pH adjusted to 7. Bacterial colonies were repeatedly recultured until pure cultures were obtained. The isolated strains were stored at -20 °C in 25% (v/v) glycerol prior to their characterization and evaluation.
Methods

Molecular identification (16s rDNA)

Bacteria was pre-cultured in Luria-Bertani broth (2 mL) during 24 h at 30 °C and 150 rpm. DNA extraction was performed using the technique of Cutting and Vander (1990). Bacterial DNA was amplified using universal primers 27f (5’ AGAGTTTGATCCTGGCTCAG 3’) and 1492r (5’ GGTTACCTTGTTACGACTT 3’), which amplify the 16S rDNA coding region. The amplification reactions were performed in a total volume of 50 μL containing 300 ng of DNA, 5 μL of PCR buffer, 2.5 U of polymerase, 1 μL (100 pmol) of each primer, 3 μL MgCl₂ (1.5 mM), 1 μL of dNTP mixture (200 µM), and H₂O Milli-Q. The amplification reactions were performed in a thermal cycler (BioRad® T100, Hercules, CA, USA) using the conditions reported by Bailón-Salas et al. (2017). All amplicons were analyzed on 1% agarose gels to confirm the fragment size; PCR products were purified with the (Promega, Fitchburg, WI, USA).

Clone library, sequencing, and phylogenetic analysis

Each amplicon was ligated into the plasmid pGEM-T Easy Vector and then transformed into E. coli DH5α competent cells according to the manufacturer's specifications. Plasmid DNA was extracted using PureYield™ Plasmid Miniprep (Promega) and sequenced by the DNA Synthesis and Sequencing Unit of the Institute of Biotechnology (UNAM, Morelos, Mexico). The sequences were compared with 16S rRNA gene sequences published in the National Center for Biotechnology Information DNA database using Basic Local Alignment Search Tool (BLAST) (Bethesda, MD, USA) and were aligned using ClustalW of BioEdit version 7.2.5 (Hall 1999). Evolutionary analyses were conducted in a MEGA7 (Kumar et al. 2016) using the maximum likelihood method (1000 iterations) (Kimura 1980). The nucleotide sequences were deposited in the GenBank public database under the accession numbers (MG977715.1, MG977716.1, MG977717.1, MG977718.1, MG977719.1, MG977720.1, MG977721.1, MG977722.1, MG977723.1, MG977724.1, MG977725.1, MG977726.1, MG977727.1, MG977728.1, and MG977729.1).

Enzyme assays

Semi-quantitative assessment was performed on over 15 colonies isolated from the aerated lagoon of a pulp and paper mill effluent. All bacterial isolates were cultured in 2 mL of Luria-Bertani liquid agar (LB) and incubated at 30 °C for 24 h. Two microliters of overnight growth culture from each isolate bacterium was spot plated to determine each enzymatic activity. This procedure was performed in triplicate. Agar plates were incubated at 30 °C for 24 h, and enzymatic tests were performed on the solid medium supplemented with different substrates.

Cellulolytic activity determination

The detection of the cellulolytic potential of the bacterium isolates was evaluated using the Congo red method as described by Teather and Wood (1982). Drip seeding was carried out on cellulose agar (granular, Sigma, Darmstadt, Germany) at 1% (w/v). After incubation, 1% Congo red (w/v) was added as a developer to the colonies present in the media. After 15 min, the excess was removed, and 0.1 M NaCl was added and allowed to stand for 15 min (Wood et al. 1988). The cellulolytic activity or hydrolysis capacity was detected by the zone around the colonies after Congo red staining. The enzymatic index (EI) was calculated according to Bortolazzo (2011), where the diameter of hydrolysis is
divided by the diameter growth of the colony.

Lipolytic activity
The phospholipase activity was detected using egg-yolk agar (10%) suspension as a substrate. Lipase production is positive when clearing zones are formed around the colonies (Strausberg et al. 1995; Varadarajan et al. 2005). The growth of the colony and the halo of degradation around it were measured after 24 h, obtaining a percentage of degradation. Lipolytic activity ($P_z$) was calculated according to Dagdeviren et al. (2005). The $P_z$ values were evaluated as follows: 1.0, negative; 0.99 to 0.9, weak; 0.89 to 0.8, mild; 0.79 to 0.7, relatively strong; and $< 0.69$, very strong activity.

Amylolytic activity
To measure starch degradation, plates of LB agar were supplemented with starch (soluble starch, 10 mg, agar, 1.7 g, distilled water, up to 100 mL). After incubation, the amylolytic activity was detected by the formation of translucent halos around the colonies after the addition of lugol solution (Madigan et al. 2015).

RESULTS AND DISCUSSION

Molecular Identification
Fifteen bacteria of pure cultures were isolated from wastewater samples collected from a pulp and paper mill wastewater. Phylogenetic analysis showed that the isolated bacteria belonged to the Firmicutes, Bacteroidetes, and Actinobacteria groups (Fig. 1). The tree with the highest log-likelihood (-5260.6783) is shown. The initial tree(s) for the heuristic search were obtained automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then the topology with superior log likelihood value was selected. The tree was drawn to scale with branch lengths measured in the number of substitutions per site. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura 1980). Horizontal distances indicate evolutionary relatedness, and the bar represents 0.05 base changes per nucleotide position.

The analysis of the cloned sequences submitted to the BLAST search is shown in Table 1. Bacillus genera was the dominant bacteria detected. This matches the isolates identified in pulp and paper mill sludge (Chandra et al. 2007; Yang et al. 2008; Mishra and Thakur 2010; Hooda et al. 2015) and in paper industry waste (Shaikh et al. 2013).

Chryseobacterium sp., a filamentous bacterium, was isolated from paper mill slimes (Oppong et al. 2003) and sludge from a kraft pulp plant (Karn et al. 2015). M. sediminis a psychro-tolerant, thermos-tolerant, halotolerant, and alkali-tolerant bacterium has only been isolated from deep-sea sediment (Yu et al. 2013).

Determination of Enzymatic Activities
Individual bacterium isolates were assessed for their ability to hydrolyze cellulose, lipids, and starch.
Fig. 1. Bacterial phylogenetic tree based on 16S rDNA gene sequences from the clone library

Table 1. Molecular Identification of the Isolates.

<table>
<thead>
<tr>
<th>ID</th>
<th>Phylogenetic Affiliation</th>
<th>Organism</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Firmicutes</td>
<td>B. subtilis KY206830.1</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>Firmicutes</td>
<td>B. megaterium MG011586.1</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>Firmicutes</td>
<td>B. licheniformis MG283323.1</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>Firmicutes</td>
<td>B. subtilis subsp. Subtilis KY886249.1</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>Firmicutes</td>
<td>B. licheniformis MF045813.1</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>Firmicutes</td>
<td>B. subtilis subsp. Subtilis KY886249.1</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>Firmicutes</td>
<td>B. cereus KY495215.1</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>Bacteroidetes</td>
<td>C. daecheongense NR_114019.1</td>
<td>99</td>
</tr>
<tr>
<td>9</td>
<td>Firmicutes</td>
<td>B. thuringiensis MG722793.1</td>
<td>99</td>
</tr>
<tr>
<td>10</td>
<td>Actinobacteria</td>
<td>M. sediminis KP282791.1</td>
<td>99</td>
</tr>
<tr>
<td>11</td>
<td>Firmicutes</td>
<td>Bacillus pumilus MF187646.1</td>
<td>100</td>
</tr>
<tr>
<td>12</td>
<td>Firmicutes</td>
<td>B. licheniformis MF045813.1</td>
<td>100</td>
</tr>
<tr>
<td>13</td>
<td>Firmicutes</td>
<td>B. subtilis KY072760.1</td>
<td>100</td>
</tr>
<tr>
<td>14</td>
<td>Firmicutes</td>
<td>B. pumilus MF187646.1</td>
<td>100</td>
</tr>
<tr>
<td>15</td>
<td>Firmicutes</td>
<td>B. cereus HE660037.1</td>
<td>100</td>
</tr>
</tbody>
</table>
Detection of Cellulolytic Activity

The endoglucanase activity is represented by the EI of cellulase activity in Fig. 2 and Fig. 3. *B. cereus* was a more potent bacterium in terms of hydrolysis of CMC (6.27 to 7.92). This coincides with reports from Xia et al. (2008) and Gao et al. (2008), where various samples from different sources were compared for their cellulolytic activity of cellulases. The hydrolysis halo diameters (1.47 cm, 24 h) were greater than that from isolated fungi (1.3 cm, 48 h) from the same study lagoon (Ordaz-Díaz et al. 2016). These enzymes have great potential to be used principally in recycled kraft pulps of paper production (Oksanen et al. 2000), in biofuel production (Srivastava et al. 2017), textile processing (Madhu and Chakraborty 2017), and food industry (Bamforth 2009).

![Interval plot of EI of cellulase activity from different isolates](image)

**Fig. 2.** Interval plot of EI of cellulase activity from different isolates

![Carboxymethyl cellulose digestion assay results](image)

**Fig. 3.** Carboxymethyl cellulose digestion assay results. Petri dishes containing CMC were colored with Congo red. The strains are identified by their ID numbers in Table 1.

There was similar production (0.80 to 1.14) in *B. subtilis subsp. subtilis*, *C. daecheongense*, *M. sediminis*, and one strain *B. licheniformis*. Low EI of cellulase activity (0.14 to 0.30) was found in *B. subtilis*, *B. thuringiensis*, *B. pumilus*, and two strains of *B.*
liceniformis.

Some microorganisms from extreme habitats have a capacity of environmental adaptation through modulation of enzymatic function (Brooks et al. 2010). This explains why B. cereus isolated from cheese does not exhibit cellulytic activity (Molva et al. 2009) and why in this work no CMCase activity was observed in B. megaterium.

**Determination of Lipolytic Activity**

The bacterial growth is shown on agar plates/egg yolk in Fig. 4. The bacterial colonies presented bright and iridescent halos, which detected the presence of lipases. Strains 7 (B. cereus, $P_z = 0.28$), 15 (B. cereus, $P_z = 0.37$), and 9 (B. thuringiensis, $P_z = 0.49$) were very strong producers. Strain 8 (C. daecheongense) was a mild producer ($P_z = 0.86$).

*Bacillus* species have a lipolytic system that is well-suited for biotechnological applications (Jaeger et al. 1999). Vasiie et al. (2016) recognized *Bacillus cereus* as the best lipase-producing bacteria of all isolates studied. The extracellular lipase of *Bacillus subtilis* (Ma et al. 2018) was reported. Additionally, Aboulwafa et al. (2016) reported that *B. thuringiensis* is a strong producer ($P_z = 0.49$ in the current study). Genes encoding degradative lipases have been studied (Dubois et al. 2012; Slamti et al. 2014). These bacterial lipases could be applied in pulp and paper manufacture (Gutiérrez et al. 2009) and in wastewater treatment (Hachemi et al. 2017).

![Fig. 4. Phospholipase activity in egg yolk agar of strains. The strains are identified by their ID numbers in Table 1.](image)

**Fig. 4.** Phospholipase activity in egg yolk agar of strains. The strains are identified by their ID numbers in Table 1.

![Fig. 5. The amylolytic activity at 30 °C](image)

**Fig. 5.** The amylolytic activity at 30 °C

**Determination of Amylolytic Activity**

In Fig. 5 the clear zone around the bacterial colony confirms the secretion of amylolytic enzymes by the isolates 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 13, 14, and 15. The amylolytic activity of *C. daecheongense* was the most potent for hydrolyzing starch.
Chryseobacterium species isolated from soils (Wang et al. 2011) and from organic kitchen wastes (Hasan et al. 2017) express extracellular amylases. The gene encoding the beta-amylase of B. cereus (Nanmori et al. 1993), alpha-amylase of B. subtilis (Yang et al. 2012) and in B. licheniformis (Hoshida et al. 2013) has been reported.

However, in Fig. 6 it is observed that the amylolytic activity of C. daecheongense do not have noticeable differences with strains corresponding to the Bacillus genus (isolates 1, 2, 7, 9, 11, and 12). A median production of the other strains was obtained (0.19-0.61). The Bacillus genus produces amylases that are of great interest in industrial processes due to their high thermo-stability (Goes and Sheppard 1999; Prakash and Jaiswal 2010). In this study, the amylolytic activity of B. pumilus, B. cereus, and B. thuringiensis was 61%, 55%, and 43%, respectively. The amylolytic production of B. thuringiensis isolates from cheese has been reported by Molva et al. (2009), where 27% of the isolates showed the capacity of starch hydrolysis.

![Fig. 6. Interval plot of amylolytic activity](image)

**CONCLUSIONS**

1. Fifteen bacterial strains were isolated from an aerated lagoon of a pulp and paper mill. *Bacillus* species were the dominant bacteria detected. A novel finding was the presence of *M. sediminis*.

2. Isolates 7 and 15, identified as *B. cereus*, were the most efficient producers of cellulase and lipase. *C. daecheongense* was the most effective in hydrolyzing starch.

3. The aerated lagoons of a pulp and paper mill are a promising source of bacteria with enzymes relevant to industrial applications. These bacterial isolates could be of interest for the pulp and paper wastewater treatment.
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