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

Ana M. Bailón-Salas,[a,δ] Luis A. Ordaz-Díaz,[a,b] Sergio Valle-Cervantes,[a] Javier López-Miranda,[a] Norma Urtiz-Estrada,[c] Jesús B. Páez-Lerma,[a] and Juan A. Rojas-Contreras [a,*]

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

*Contact information: a: Chemical and Biochemical Engineering Department, Durango Institute of Technology (ITD), Durango, México; b: Environmental Engineering Technology, Universidad Politécnica de Durango, Durango, México; c: Facultad de Ciencias Químicas, Universidad Juárez del Estado de Durango, Durango, México; [δ]Ph.D. Student; *Corresponding author: juanroco@hotmail.com*

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 ºC 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' AGAGTTTGAT-CCTGGCTCAG 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 $MgCl_2$ (1.5 mM), 1 µL of dNTP mixture (200 µM), and H_2O 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.

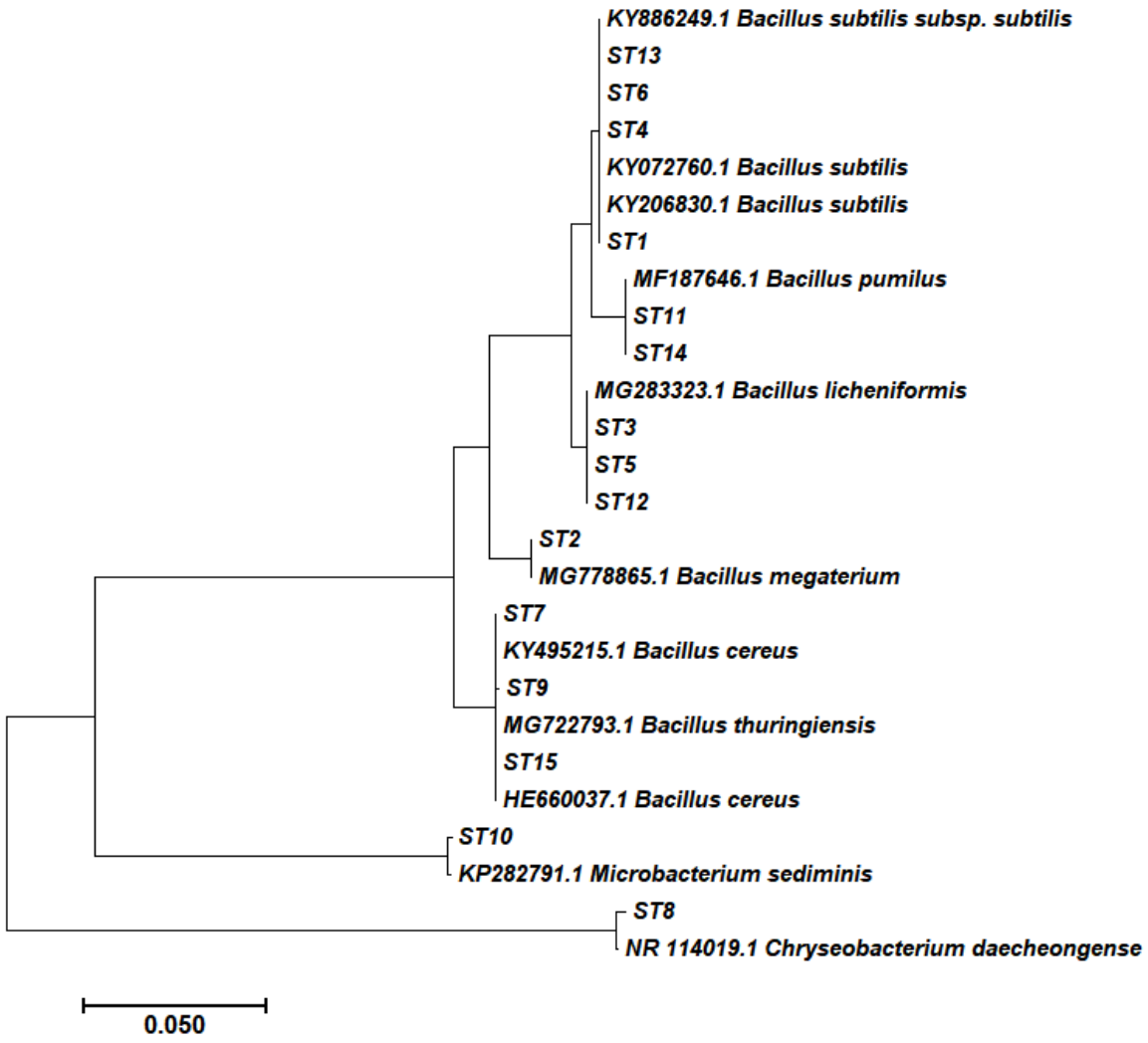

Fig. 1. Bacterial phylogenetic tree based on16S rDNA gene sequences from the clone library

Table 1. Molecular Identification of the Isolates.

ID	Phylogenetic Affiliation	Organism	Similarity (%)
1	Firmicutes	*B. subtilis* KY206830.1	100
2	Firmicutes	*B. megaterium* MG011586.1	100
3	Firmicutes	*B. licheniformis* MG283323.1	100
4	Firmicutes	*B. subtilis subsp. Subtilis* KY886249.1	100
5	Firmicutes	*B. licheniformis* MF045813.1	100
6	Firmicutes	*B. subtilis subsp. Subtilis* KY886249.1	100
7	Firmicutes	*B. cereus* KY495215.1	100
8	Bacteriodetes	*C. daecheongense* NR_114019.1	99
9	Firmicutes	*B. thuringiensis* MG722793.1	99
10	Actinobacteria	*M. sediminis* KP282791.1	99
11	Firmicutes	*Bacillus pumilus* MF187646.1	100
12	Firmicutes	*B. licheniformis* MF045813.1	100
13	Firmicutes	*B. subtilis* KY072760.1	100
14	Firmicutes	*B. pumilus* MF187646.1	100
15	Firmicutes	*B. cereus* HE660037.1	100

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-Dìaz *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).

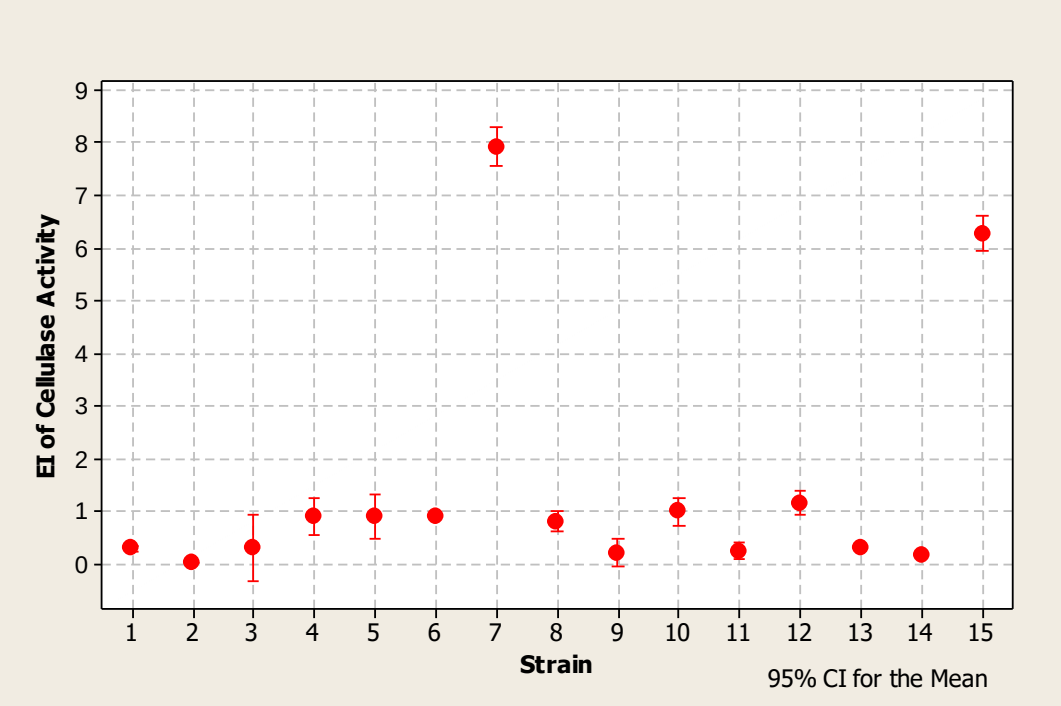

Fig. 2. Interval plot of EI of cellulase activity from different isolates

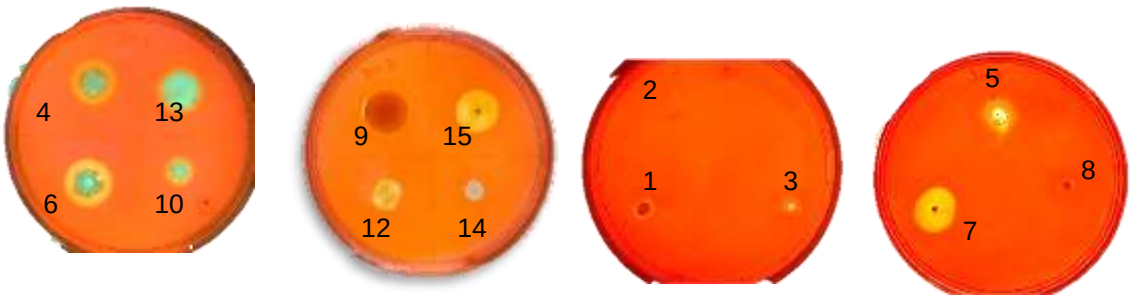

Fig. 3. Carboxymethyl cellulose digestion assay results. Petri dishes containing CMC were colored with Congo red. The strains are identified by their ID numbers in Table 1.

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.*

licheniformis.

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 cellulolytic 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). Vasiee *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.

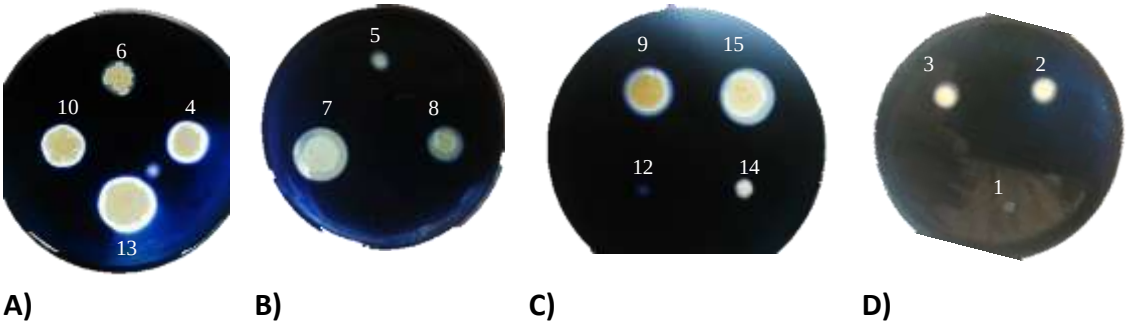

Fig. 5. The amylolytic activity at 30 °C

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.

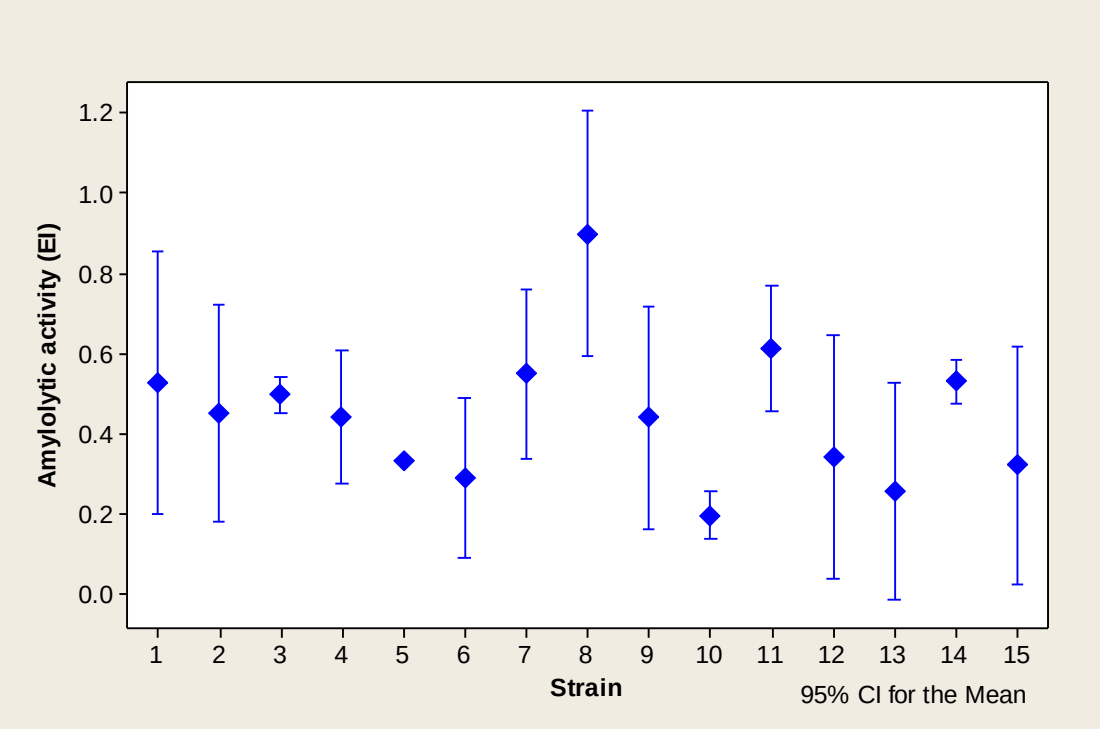

Fig. 6. Interval plot of amylolytic activity

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.

ACKNOWLEDGMENTS

The support of the Science and Technology National Council (CONACyT) was gratefully appreciated, as it was received by one of the writers for the scholarship (419668/257494).

REFERENCES CITED

Aboulwafa, M., Elleboudy, N., ElKhatib, W., and Hassouna, N. (2016). "Production and characterization of phospholipases C from some *Bacillus thuringiensis* isolates recovered from Egyptian soil," *International Journal of Biotechnology for Wellness Industries* 5(1), 10-24. DOI: 10.6000/1927-3037.2016.05.01.3

Bailón-Salas, A. M., Ordaz-Díaz, L. A., Valle-Cervantes, S., López-Miranda, J., Urtiz-Estrada, N., Páez-Lerma, J. B., de León-Mata, G. D., and Rojas-Contreras, J. A. (2017). "Bacterial diversity in two aerated lagoons of a pulp and paper effluent and their interaction with a commercial inoculum using PCR-DGGE," *BioResources* 12(3), 5487-5501. DOI: 10.15376/biores.12.3.5487-5501

Bajpai, P. (1999). "Application of enzymes in the pulp and paper industry," *Biotechnology Progress* 15(2), 147-157. DOI: 10.1021/bp990013k

Bamforth, C. W. (2009). "Current perspectives on the role of enzymes in brewing," *Journal of Cereal Science* 50(3), 353-357. DOI: 10.1016/j.jcs.2009.03.001

Bortolazzo, N. G. (2011). *Isolamento e seleção de fungos celulolíticos para hidrólise enzimática do bagaço de cana-de-açúcar*, Ph.D. Dissertation, Universidade de São Paulo, São Paulo, Brazil.

Brooks, A. N., Turkarslan, S., Beer, K. D., Yin Lo, F., and Baliga, N. S. (2010). "Adaptation of cells to new environments," *Wiley Interdisciplinary Reviews: Systems Biology and Medicine* 3(5), 544-561. DOI: 10.1002/wsbm.136

Chandra, R., Raj, A., Purohit, H. J., and Kapley, A. (2007). "Characterisation and optimisation of three potential aerobic bacterial strains for kraft lignin degradation from pulp paper waste," *Chemosphere* 67(4), 839-846. DOI: 10.1016/j.chemosphere.2006.10.011

Cutting, S. M., and Vander Horn, P. B. (1990). "Genetic analysis," in: *Molecular Biological Methods for Bacillus*, John Wiley and Sons, Sussex, UK, pp. 27-74.

Dagdeviren, M., Cerikcioglu, N., and Karavus, M. (2005). "Acid proteinase, phospholipase and adherence properties of *Candida parapsilosis* strains isolated from clinical specimens of hospitalised patients," *Mycoses* 48(5), 321-326. DOI: 10.1111/j.1439-0507.2005. 01145.x

Dubois, T., Faegri, K., Perchat, S., Lemy, C., Buisson, C., Nielsen-Leroux, C., Gohar, M., Jacques, P., Ramarao, N., Kolstø, A-B, and Lereclus, D. (2012). "Necrotrophism is a quorum-sensing-regulated lifestyle in *Bacillus thuringiensis*," *PLoS Pathogens* 8(4), e1002629. DOI: 10.1371/journal.ppat.1002629

Forster, S., Lappin-Scott, H. M., Snape, J. R., and Porter, J. (2003). "Rains, drains and active strains: Towards online assessment of wastewater bacterial communities," *Journal of Microbiological Methods* 55(3), 859-864. DOI: 10.1016/j.mimet.2003.08.004

Gao, X. A., Ju, W. T., Jung, W. J., and Park, R. D. (2008). "Purification and characterization of chitosanase from *Bacillus cereus* D-11," *Carbohydrate Polymers*

72(3), 513-520. DOI: 10.1016/j.carbpol.2007.09.025

Goes, A. P., and Sheppard, J. D. (1999). "Effect of surfactants on α-amylase production in a solid substrate fermentation process," *Journal of Chemical Technology and Biotechnology* 74(7), 709-712. DOI: 10.1002/(SICI)1097-4660(199907)74:7<709::AID-JCTB94>3.0.CO;2-C

Gratia, E., Weekers, F., Margesin, R., D'Amico, S., Thonart, P., and Feller, G. (2009). "Selection of a cold-adapted bacterium for bioremediation of wastewater at low temperatures," *Extremophiles* 13(5), 763-768. DOI: 10.1007/s00792-009-0264-0

Gutiérrez, A., del Río, J. C., and Martínez, A. T. (2009). "Microbial and enzymatic control of pitch in the pulp and paper industry," *Applied Microbiology and Biotechnology* 82(6), 1005-1018. DOI: 10.1007/s00253-009-1905-z

Hachemi, L., Benattouche, Z., and Belgherras, M. E. (2017). "Lipolytic bacteria use as bio-decontaminating natural in the water purification stations," *International Journal of Biological Macromolecules* 105, 873-878. DOI: 10.1016/j.ijbiomac.2017.07.106

Hall, T. A. (1999). "BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT," *Nucleic Acids Symposium Series* 41, 95-98.

Hansen, G. H., Lübeck, M., Frisvad, J. C., Lübeck, P. S., and Andersen, B. (2015). "Production of cellulolytic enzymes from ascomycetes: Comparison of solid state and submerged fermentation," *Process Biochemistry* 50(9), 1327-1341. DOI: 10.1016/j.procbio.2015.05.017

Hasan, M. M., Marzan, L. W., Hosna, A., Hakim, A., and Azad, A. K. (2017). "Optimization of some fermentation conditions for the production of extracellular amylases by using *Chryseobacterium* and *Bacillus* isolates from organic kitchen wastes," *Journal of Genetic Engineering and Biotechnology* 15(1), 59-68. DOI: 10.1016/j.jgeb.2017.02.009

Hooda, R., Bhardwaj, N. K., and Singh, P. (2015). "Screening and identification of ligninolytic bacteria for the treatment of pulp and paper mill effluent," *Water, Air, and Soil Pollution* 226(9), 305. DOI: 10.1007/s11270-015-2535-y

Hoshida, H., Fujita, T., Cha-aim, K., and Akada, R. (2013). "N-glycosylation deficiency enhanced heterologous production of a *Bacillus licheniformis* thermostable α-amylase in *Saccharomyces cerevisiae*," *Applied Microbiology and Biotechnology* 97(12), 5473-5482. DOI: 10.1007/s00253-012-4582-2

Hough, D. W., and Danson, M. J. (1999). "Extremozymes," *Current Opinion in Chemical Biology 3*(1), 39-46. DOI: 10.1016/S1367-5931(99)80008-8

Hugenholtz, P. (2002). "Exploring prokaryotic diversity in the genomic era," *Genome Biology* 3(2), 1-8. DOI: 10.1186/gb-2002-3-2-reviews0003

Jaeger, K. E., Dijkstra, B. W., and Reetz, M. T. (1999). "Bacterial biocatalysts: Molecular biology, three-dimensional structures, and biotechnological applications of lipases," *Annual Reviews in Microbiology* 53(1), 315-351. DOI: 10.1146/annurev.micro.53.1.315

Karam, J., and Nicell, J. A. (1997). "Potential applications of enzymes in waste treatment," *Journal of Chemical Technology and Biotechnology* 69(2), 141-153. DOI: 10.1002/(SICI)1097-4660(199706)69:2<141::AID-JCTB694>3.0.CO;2-U

Karn, S. K., Kumari, S., and Chakrabarti, S. K. (2015). "Bio-removal of chlorophenols from industrial effluents in open bioreactor system," *Journal of Chemistry & Applied Biochemistry* 2(1), 108.

Karrasch, B., Parra, O., Cid, H., Mehrens, M., Pacheco, P., Urrutia, R., Valdovinos, C.,

and Zaror, C. (2006). "Effects of pulp and paper mill effluents on the microplankton and microbial self-purification capabilities of the Biobío river, Chile," *Science of the Total Environment* 359(1), 194-208. DOI: 10.1016/j.scitotenv.2005.03.029

Kimura, M. (1980). "A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences," *Journal of Molecular Evolution* 16(2), 111-120. DOI: 10.1007/BF01731581

Kulshreshtha, N. M., Kumar A., Dhall P., Gupta, S., Bisht, G., Pasha, S., and Kumar, R. (2010). "Neutralization of alkaline industrial wastewaters using *Exiguobacterium sp.*," *International Biodeterioration and Biodegradation* 64(3), 191-196. DOI: 10.1016/j.ibiod.2010.01.003

Kumar, S., Stecher, G., and Tamura, K. (2016). "MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets," *Molecular Biology and Evolution* 33(7), 1870-1874. DOI: 10.1093/molbev/msw054

Kyllönen, H. L., Lappi, M. K., Thun, R. T., and Mustranta, A. H. (1988). "Treatment and characterization of biological sludges from the pulp and paper industry," *Water Science and Technology* 20(1), 183-192.

Littlechild, J. A. (2015). "Enzymes from extreme environments and their industrial applications," *Frontiers in Bioengineering and Biotechnology* 3, 161. DOI: 10.3389/fbioe.2015.00161

Liu, X., and Kokare, C. (2017). "Microbial enzymes of use in industry," *Biotechnology of Microbial Enzymes* 2017, 267-298. DOI: 10.1016/B978-0-12-803725-6.00011-X

Ma, R. J., Wang, Y. H., Liu, L., Bai, L. L., and Ban, R. (2018). "Production enhancement of the extracellular lipase LipA in *Bacillus subtilis*: Effects of expression system and Sec pathway components," *Protein expression and purification*, 142, 81-87. DOI: 10.1016/j.pep.2017.09.011

Madigan, M. T., Martinko, J. M., Bender, K. S., Buckley, D. H., and Stahl, D. A. (2015). *Brock: Biología de los Microorganismos* (14th Ed.), Pearson Educación, Madrid, Spain.

Martínez-Martínez, M., Bargiela, R., and Ferrer, M. (2017). "Metagenomics and the search for industrial enzymes," *Biotechnology of Microbial Enzymes* 2017, 167-184. DOI: 10.1016/B978-0-12-803725-6.00007-8

Mishra, M., and Thakur, I. S. (2010). "Isolation and characterization of alkalotolerant bacteria and optimization of process parameters for decolorization and detoxification of pulp and paper mill effluent by Taguchi approach," *Biodegradation* 21(6), 967-978. DOI: 10.1007/s10532-010-9356-x

Molina-Muñoz, M., Poyatos, J. M., Rodelas, B., Pozo, C., Manzanera, M., Hontoria, E., and Gonzalez-Lopez, J. (2010). "Microbial enzymatic activities in a pilot-scale MBR experimental plant under different working conditions," *Bioresource Technology* 101, 696-704. DOI: 10.1016/j.biortech.2009.08.071

Molva, C., Sudagidan, M., and Okuklu, B. (2009). "Extracellular enzyme production and enterotoxigenic gene profiles of *Bacillus cereus* and *Bacillus thuringiensis* strains isolated from cheese in Turkey," *Food Control* 20(9), 829-834. DOI: 10.1016/j.foodcont.2008.10.016

Nanmori, T., Nagai, M., Shimizu, Y., Shinke, R., and Mikami, B. (1993). "Cloning of the beta-amylase gene from *Bacillus cereus* and characteristics of the primary structure of the enzyme," *Applied and Environmental Microbiology* 59(2), 623-627.

Oksanen, T., Pere, J., Paavilainen, L., Buchert, J., and Viikari, L. (2000). "Treatment of recycled kraft pulps with *Trichoderma reesei* hemicellulases and cellulases*,"*

Journal of Biotechnology 78(1), 39-48. DOI: 10.1016/S0168-1656(99)00232-1

Oppong, D., King, V. M., and Bowen, J. A. (2003). "Isolation and characterization of filamentous bacteria from paper mill slimes," *International Biodeterioration & Biodegradation* 52(2), 53-62. DOI: 10.1016/S0964-8305(02)00174-9

Ordaz-Díaz, L. A., Rojas-Contreras, J. A., Flores-Vichi, F., Flores-Villegas, M. Y., Álvarez-Álvarez, C., Velasco-Vázquez, P., and Bailón-Salas, A. M. (2016). "Quantification of endoglucanase activity based on carboxymethyl cellulose in four fungi isolated from an aerated lagoon in a pulp and paper mill," *BioResources* 11(3), 7781-7789. DOI: 10.15376/biores.11.3.7781-7789

Persson, I., Tjerneld, F., and Hahn-Hägerdal, B. (1991). "Fungal cellulolytic enzyme production: A review," *Process Biochemistry* 26(2), 65-74. DOI: 10.1016/0032-9592(91)80019-L

Prakash, O., and Jaiswal, N. (2010). "α-Amylase: An ideal representative of thermostable enzymes," *Applied Biochemistry and Biotechnology* 160(8), 2401-2414. DOI: 10.1007/s12010-009-8735-4

Rondon, M. R., Goodman, R. M., and Handelsman, J. (1999). "The Earth's bounty: Assessing and accessing soil microbial diversity," *Trends in Biotechnology* 17(10), 403-409. DOI: 10.1016/S0167-7799(99)01352-9

Shaikh, N. M., Patel, A. A., Mehta, S. A., and Patel, N. D. (2013). "Isolation and screening of cellulolytic bacteria inhabiting different environment and optimization of cellulase production," *Universal Journal of Environmental Research and Technology* 3(1), 39-49.

Slamti, L., Perchat, S., Huillet, E., and Lereclus, D. (2014). "Quorum sensing in *Bacillus thuringiensis* is required for completion of a full infectious cycle in the insect," *Toxins* 6(8), 2239-2255. DOI: 10.3390/toxins6082239

Srivastava, A., D McMahon, K., Stepanauskas, R., and Grossart, H. P. (2016). "De novo synthesis and functional analysis of the phosphatase-encoding gene acI-B of uncultured Actinobacteria from Lake Stechlin (NE Germany)," *International Microbiology* 19(1), 39-47. DOI: 10.2436/20.1501.01.262

Srivastava, N., Srivastava, M., Mishra, P. K., Gupta, V. K., Molina, G., Rodriguez-Couto, S., Manikanta, A., and Ramteke, P. W. (2017). "Applications of fungal cellulases in biofuel production: Advances and limitations," *Renewable and Sustainable Energy Reviews* 82, 2379-2386. DOI: 10.1016/j.rser.2017.08.074

Strausberg, S. L., Alexander, P. A., Gallagher, D. T., Gilliland, G. L., Barnett, B. L., and Bryan, P. N. (1995). "Directed evolution of a subtilisin with calcium-independent stability," *Nature Biotechnology* 13(7), 669-673. DOI: 10.1038/nbt0795-669

Streit, W. R., and Schmitz, R. A. (2004). "Metagenomics–The key to the uncultured microbes," *Current Opinion in Microbiology* 7(5), 492-498. DOI: 10.1016/j.mib.2004.08.002

Teather, R. M., and Wood, P. J. (1982). "Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen," *Applied and Environmental Microbiology* 43(4), 777-780.

Thompson, G., Swain, J., Kay, M., and Forster, C. F. (2001). "The treatment of pulp and paper mill effluent: A review," *Bioresource Technology* 77(3), 275-286. DOI: 10.1016/S0960-8524(00)00060-2

Varadarajan, N., Gam, J., Olsen, M. J., Georgiou, G., and Iverson, B. L. (2005). "Engineering of protease variants exhibiting high catalytic activity and exquisite substrate selectivity," *Proceedings of the National Academy of Sciences of the*

United States of America 102(19), 6855-6860. DOI: 10.1073/pnas.0500063102

Vasiee, A., Behbahani, B. A., Yazdi, F. T., and Moradi, S. (2016). "Optimization of the production conditions of the lipase produced by bacillus cereus from rice flour through Plackett-Burman design (PBD) and response surface methodology (RSM)," *Microbial Pathogenesis* 101, 36-43. DOI: 10.1016/j.micpath.2016.10.020

Wang, S. L., Liang, Y. C., and Liang, T. W. (2011). "Purification and characterization of a novel alkali-stable α-amylase from *Chryseobacterium taeanense* TKU001, and application in antioxidant and prebiotic," *Process Biochemistry* 46(3), 745-750. DOI: 10.1016/j.procbio.2010.11.022

Wood, P. J., Erfle, J. D., and Teather, R. M. (1988). "Use of complex formation between Congo red and polysaccharides in detection and assay of polysaccharide hydrolases," *Methods in Enzymology*, 59-74. DOI: 10.1016/0076-6879(88)60107-8

Xia, W., Liu, P., and Liu, J. (2008). "Advance in chitosan hydrolysis by non-specific cellulases," *Bioresource Technology* 99(15), 6751-6762. DOI: 10.1016/j.biortech.2008.01.011

Yang, C., Cao, G., Li, Y., Zhang, X., Ren, H., Wang, X., Feng, J., Zhao, L., and Xu, P. (2008). "A constructed alkaline consortium and its dynamics in treating alkaline black liquor with very high pollution load," *PloS ONE* 3(11), e3777. DOI: 10.1371/journal.pone.0003777

Yang, H., Liu, L., Li, J., Du, G., and Chen, J. (2012). "Cloning, heterologous expression, and comparative characterization of a mesophilic α-amylase gene from *Bacillus subtilis* JN16 in *Escherichia coli*," *Annals of microbiology* 62(3), 1219-1226. DOI: 10.1007/s13213-011-0364-9

Yu, L., Lai, Q., Yi, Z., Zhang, L., Huang, Y., Gu, L., and Tang, X. (2013). "*Microbacterium sediminis* sp. nov., a psychrotolerant, thermotolerant, halotolerant and alkalitolerant actinomycete isolated from deep-sea sediment," *International Journal of Systematic and Evolutionary Microbiology* 63(1), 25-30. DOI: 10.1099/ijs.0.029652-0

Article submitted: February 27, 2018; Peer review completed: May 1, 2018; Revised version received: May 9, 2018; Accepted; May 10, 2018; Published: May 15, 2018.
DOI: 10.15376/biores.13.3.5052-5064