ISOLATING AND SELECTING MICROORGANISMS TO OBTAIN CELLULOSE SUSTAINABLY

By

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Abstract

ACTUAL chemical processes able to obtain cellulose from sugarcane bagasse are very toxic and pollutant. The objective of this work was to find native microorganisms able to delignify bagasse helping to raise its cellulose content, without affecting actual fibres, in order to obtain higher quality paper. Advantages of biological delignification compared to chemical methods are: softer reaction conditions, higher product yields and less energy consumption. Another advantage is the improvement in the paper's mechanical properties such as tensile strength. For isolation and identification of the microorganisms, such as bacteria and fungi, sugarcane bagasse was used as the only substrate, which was obtained from a Mexican sugar mill. The plate dilution and enrichment culture techniques were applied. Six native strains of fungi and four of bacteria have been isolated, some of them with potential capacity to degrade the lignin which is due to lignolytic extracellular enzyme production, yielding laccases and peroxidases. Obtained results indicate that biotechnological lignin degradation is possible using native microorganisms from sugarcane bagasse thus eliminating chemical processes. The biological delignification of the sugarcane bagasse for obtaining cellulose could be considered as a sustainable process not only because of the recycling of this by-product but also because it is environmentally friendly.

Introduction

Sugarcane bagasse is a by-product of the sugar industry consisting principally of cellulose, hemicellulose and lignin. It is the fibrous residue of sugarcane after the juice has been extracted. In Mexico, more than 95% of the bagasse is used by the sugar factories as fuel for the boilers. In recent years, alternative and more profitable uses of bagasse are being investigated.

Bagasse has been used for the production of enzymes, amino acids, drugs, ethanol, and single-cell protein as animal feed after treating it with a large variety of microorganisms including bacteria, yeasts, and fungi, among which *Basidiomycetes* are preferred (Eriksson, 1990). However, such bio-processes require only small quantities of substrate and would not utilise all available bagasse (Xin *et al.*, 2002).

Due to increasing restraints on forest harvesting, the use of agro-based residues for pulp and paper production has been steadily increasing in recent years (Bustamante *et al.*, 1999). The sugarcane bagasse is a biomass that can be used as raw material for pulp and paper production, which in turn yields new paper, dissolvable pulp and cellulose-based filter paper for soft drinks and other liquids (Valdes, 2007).

Lignin is the most abundant renewable source of aromatic polymers in nature, and its degradation is therefore of general significance for the global carbon cycle. It is chemically recalcitrant to breakdown by most organisms because of the complex, heterogeneous structure. The most efficient and most investigated lignin-degrading microorganisms are the wood-decaying *Basidiomycetes* causing white-rot. They contain the extracellular oxidative enzymes laccases and peroxidases (Gao *et al.*, 1997).

The major groups of lignolytic enzymes include lignin peroxidases, manganese peroxidases, versatile peroxidases, and laccases. The peroxidases are heme-containing enzymes with catalytic cycles that involve the activation by H_2O_2 and substrate reduction of compound I and compound II intermediates. Lignin peroxidases have the unique ability to catalyse oxidative cleavage of C–C bonds and ether (C–O–C) bonds in non-phenolic aromatic substrates of high redox potential. Manganese peroxidases oxidise Mn(II) to Mn(III), which facilitates the degradation of phenolic compounds or, in turn, oxidises a second mediator for the breakdown of non-phenolic compounds. Versatile peroxidases are hybrids of lignin peroxidase and manganese peroxidase with a bifunctional characteristic. Laccases are multi-copper-containing proteins that catalyse the oxidation of phenolic substrates with concomitant reduction of molecular oxygen to water (Wong, 2009).

Breccia *et al.* (1997) screened several white-rot fungi to degrade long-fibre bagasse aiming at bio-pulping and found that about 16% of the lignin was removed. Biological pulping has the potential to reduce energy costs and environmental impact compared to traditional pulping operations (Breen and Singleton, 1999).

The objective of this work was isolating and identifying the strains of native microorganisms which assist in delignifying bagasse and as a result raise its cellulose content. The enrichment culturing technique was used. Thus, cellulose would become a by-product of the cane sugar industry. The present study describes a simple system for isolating and screening of microorganisms, either fungi or bacteria, which have lignolytic activities.

Materials and methods

Sample source

The sugarcane bagasse samples were provided by Ingenio San José de Abajo, from Veracruz, México.

Culture media

- Saline medium for enrichment cultures. Composition in g/L: KNO_3 1.0; $FeCl_3$ 0.02; $MgSO_4$ 0.2; NaCl 0.1; $CaCl_2$ 0.1; K_2HPO_4 1.0 and yeast extract 0.05. The pH of the medium was adjusted to 6.3 with a 0.1 N HCl solution.
- Saline Agar. This medium contains the same components as the saline medium, but is complemented with 1.5% (w/v) bacteriological agar. The pH of the medium was also adjusted to 6.3 with a 0.1 N HCl solution.
- Malt Extract Agar. Malt extract Agar (Difco). Sabouraud Dextrose Agar (BD Bioxon), and Nutritive Agar (BD Bioxon).

Microorganisms isolation

The isolation of the microorganisms, bacteria as well as fungi, was done from sugarcane bagasse applying enrichment culturing.

Sugarcane bagasse was washed with water at 60°C in order to remove the sugar. It was used as the only substrate. Then, 1.0 g of this bagasse was added into a 250 mL Erlenmeyer flask which contained 125.0 mL of the saline medium. It was cultured by shaking for three days at 25°C and 180 rpm. After being incubated for three days, 12.0 mL of the supernatant was transferred to an Erlenmeyer flask of 250 mL containing 125 mL of fresh medium, which was cultured at the same above described conditions. This process was repeated three times.

Isolation and identification of microorganisms

After three transfers, 0.1 mL of sample from the recuperated medium was spread on saline agar plates and incubated at 26°C for 24 hours. For isolation, the obtained colonies were inoculated on to nutritive agar plates if they were bacteria or on malt extract agar and Sabouraud dextrose agar if they were fungi. The plates were incubated for another 24 h at 35°C and for 72 h at 26°C, respectively.

The fungi were primarily identified based on their characteristics of colonial morphology and structure. For the structure identification, a micro-culture from each isolated colony was prepared, which was incubated at 26°C until sporulation. Final fungi identification was done after staining with lactophenol-cotton blue and observation under the microscope. Photos were taken.

Bacteria could be identified based on their colonial morphology and biological characteristics, and physiological and biochemical tests including Gram reaction (Buchanan and Gibbons, 1974).

Results

The results show ten strains of microorganisms, which were obtained by the enrichment culture technique using sugarcane bagasse as the only source of carbon and energy. Of the ten strains isolated, six were fungi, which are named F-1 through F-6; the others were bacteria and are named B-1 through B-4. Figure 1 shows the macroscopic and microscopic characteristics of isolated fungi and their corresponding identification. The morphology and Gram reaction of the isolated bacteria are shown in Figure 2. Table 1 shows the physiological and biochemical characteristics of strains of isolated bacteria.

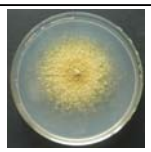
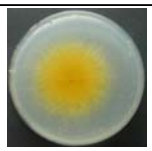
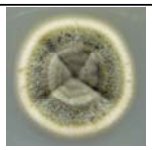
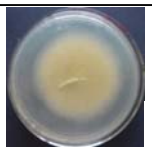
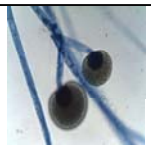
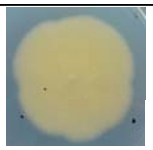
Strain	Obverse	Reverse	Micrography
F-1 *Penicllium sp.*			
F-2 *Penicllium sp.*			
F-3 *Fusarium sp.*			
F-4 *Cladosporium sp.*			
F-5 *Mucor sp.*			
F-6 *Geotrichum sp.*			

Fig. 1—Macroscopic and microscopic characterisation for fungi isolated and identified.

Table 1—Physiological and biochemical characteristics of strains of bacteria.

Strain	B-1	B-2	B-3	B-4
Shape	Rod	Rod	Rod	Rod
Gram reaction	+	+	+	+
Mobility or not	+	+	+	+
Methyl red	+	–	+	+
Voges Proskauer	+	+	+	+
Catalase	+	+	+	
Oxidase	–	+	–	+
Starch hydrolysis	+	+	+	+
Glucose fermentation	+	+	+	+
Citrate utilisation	+	+	+	+

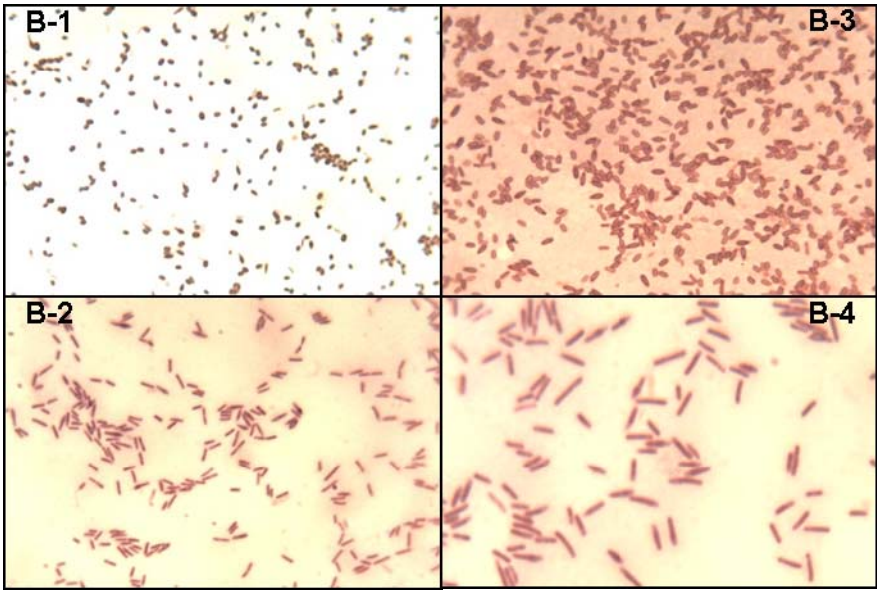


Fig. 2—Micrographics that show the morphology and Gram reaction of the bacteria isolated, where B-1, B-3 and B-4 have been identified as *Bacillus subtilis* and B-2 as *Bacillus sp*.

Conclusions

There are reports in the literature on several identified lignolytic fungi and bacteria suitable for biotechnological processes on bagasse permitting diversification of the sugar industry (Meza *et al.*, 2006; Ramos *et al.*, 2001; Viñals Verde *et al.*, 2006; Xin *et al.*, 2002).

In this study, six different native strains of fungi were isolated and identified: *Penicillium sp.* (two strains), *Fusarium sp., Cladosporium sp., Mucor sp.* and *Geotrichum sp.;* also four bacteria, all of the gender *Bacillus*. The production of lignolytic enzymes by these microorganisms will be tested later with liquid cultures.

It is also intended continuing this work to isolate fungi of the *Basidiomycetes* group, which are reported as having higher lignolytic capacity.

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L'ISOLEMENT ET LA SÉLECTION DES MICRO-ORGANISMES POUR LA PRODUCTION DURABLE DE CELLULOSE

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MOTS-CLÉS: Champignons et Bactéries Lignolytiques,
Production Biotechnologique de Cellulose,
Bagasses de la Canne à Sucre, Canne à Sucre.

Résumé

LES PROCESSUS chimiques courants, capables d'obtenir la cellulose à partir de la bagasse de canne à sucre sont très toxiques et polluants. L'objectif de ce travail était de trouver des micro-organismes natifs capables de delignify bagasse contribuant à augmenter son contenu de cellulose, sans affecter les fibres réelles, afin d'obtenir des papiers de qualité supérieure.Les avantages de la délignification biologique par rapport aux méthodes chimiques sont : conditions de réaction plus douces, hausse des rendements de produit et moins de consommation d'énergie. Un avantage supplémentaire est l'amélioration des propriétés mécaniques du papier telle que la traction. Pour l'isolement et identification des micro-organismes, comme les bactéries et champignons, seule la bagasse de canne à sucre a été utilisée comme unique substrat, obtenu à partir d'une sucrerie mexicaine. Les techniques de culture de dilution et d'enrichissement de plaque ont été appliquées. Six souches de champignons et quatre de bactéries à l'état naturel ont été isolées, certaines d'entre elles avec une capacité potentielle de dégrader la lignine de la cellulose qui est due à la production d'enzymes extracellulaires lignolytiques, produisant des laccases et peroxidases. Les résultats obtenus indiquent que la dégradation de la lignine par biotechnologie est possible à l'aide de micro-organismes trouvés de la bagasse de canne à sucre éliminant ainsi les processus chimiques. La délignification biologique de la bagasse de canne à sucre pour l'obtention de cellulose pourrait être considérée comme un processus durable non seulement en raison du recyclage de ce sous-produit mais aussi parce qu'il est plus respectueux de l'environnement.

AISLAR Y SELECCIONAR MICROORGANISMOS PARA OBTENER CELULOSA SOSTENIBLE

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PALABRAS CLAVE: Hongos y Bacterias Lignolíticas,
Producción Biotecnológica de Celulosa,
Bagazo de la Caña de Azúcar.

Resumen

LOS PROCESOS existentes capaces de obtener celulosa del bagazo de la caña de azúcar son muy tóxicos y contaminantes. El objetivo de este trabajo fue encontrar nativos microorganismos capaces de delignify bagazo ayudando a elevar su contenido de celulosa, sin que ello afecte a fibras reales, con el fin de obtener mayor papel de calidad. Las ventajas de la delignificación biológica en comparación con los métodos químicos son condiciones de reacción más suaves, mayores rendimientos de productos y menores consumos de energía. Otra ventaja es el mejoramiento de las propiedades mecánicas del papel, tales como la fuerza tensil. Para el aislamiento y al identificación de los microorganismos, tales como bacterias y hongos se empleó el bagazo de caña como único sustrato, el que se obtuvo de un Central mexicano. Se emplearon las técnicas de dilución en placas y de cultivos enriquecidos. Se aislaron seis(6) razas nativas de hongos y cuatro (4) de bacterias , algunas de ellas con capacidad d degradar la lignina a celulosa debido a la producción de una enzima lignolítica extracelular, rindiendo lacasas y peroxidasas; los resultados obtenidos indican que la degradación biotecnológica de la lignina es posible empleando microorganismos nativos del bagazo de la caña y así eliminar los procesos químicos. La delignificación biológica del bagazo de la caña de azúcar para obtener celulosa puede considerarse un proceso sostenible, no solo por el reciclado de este coproducto, sino también porque es amigable con el medio ambiente.