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Isolation of Colombian Native Bacteria and their Potential for Ethanol Production from Xylose and Glucose

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The use of residual lignocellulosic material from different agro-industrial processes as feedstock for the bioethanol production has been an energy alternative in the world due to its wide availability and low cost, which has posed major challenges in the processes of microbial fermentation of hexoses and pentoses (products available after hydrolysis of lignocellulosic material). The fermentation of pentoses (xylose and arabinose) is still a wide field of study. In this research, Colombian native bacteria able to transform xylose into ethanol were isolated and identified. Selected strains were identified by amplification and analysis of the 16S rRNA gene, and results showed they are related to members of genera such as *Airebacillus* sp., *Bacillus* sp., *Lysinibacillus* sp., *Enterococcus* sp., and *Citrobacter* sp. The isolated strains were characterized for the xylose and glucose consumption and product formation. The measured ethanol yield was in a range between 0,006 – 0,174 g ethanol/g sugar in xylose after 48 h. In a mixture of glucose and xylose, the results showed that the ethanol yield was in a range between 0,010 – 0,092 g ethanol/g sugar, and both substrates were consumed. Organic acids were obtained as fermentation product, which is a feature of these strains. The co-fermentation of xylose and glucose that was evidenced in the metabolism of the isolates represents an alternative for the transformation of the hemicellulosic fraction of agroindustrial wastes.

1. Introduction

The production of bioethanol using lignocellulosic material as a substrate is an energy alternative due to its low cost and high availability of raw material. This kind of material is the most abundant organic mass in the biosphere with approximately 50% of the existing biomass (Chandel et al, 2011). The yield of ethanol from this type of material is directly related to the content of polysaccharides, such as cellulose and hemicellulose, which normally constitute two thirds of the cell wall dry matter (Balat, 2011). The conversion of biomass to ethanol by the biochemical pathway involves five steps: 1) pre-treatment, 2) hydrolysis of complex carbohydrates, 3) fermentation, 4) distillation for product recovery and 5) effluent treatment (Cardona et al, 2010). All these comprise research challenges due to the technological and microbiological processes necessary for the bioconversion. This study focuses on the fermentation process involving the bacterial transformation of sugars (xylose and glucose) that are present in the composition of lignocellulosic material.
Efforts to develop fermentation processes have mainly focused on the degradation and conversion of cellulose, which typically constitutes 36-61% dry weight of vegetable originating biomass (Olson & Hahn-Häigerdal, 1996). In these cases, the glucose yeast fermentation has been successful. *Saccharomyces cerevisiae* is the common microorganism in the fermentation processes due to its high tolerance to inhibitors and ethanol concentration, no requirement of oxygen and low optimum pH (Lancheros-Castañeda et al, 2015). However, the hemicellulose composition present in this kind of raw material, which can be up to 30% (Dumon et al, 2012), is significant because of its high composition of pentoses, such as xylose and arabinose, wich are monosaccharides that can be transformed in this process, making it much more efficient.

Since the 1980s, research has been focused on finding pentose-fermenting microorganism and understanding the metabolism of xylose (Olsson & Hahn-Häigerdal, 1996), trying to overcome one of the limitations such as the bioconversion of pentose and achieve the development of an efficient and scalable process for the conversion of this material into ethanol. Bacteria have been proposed as promising microorganisms based on the wide range of substrates they can metabolize (not only monosaccharides can be fermented by bacteria but also cellulose biopolymers and others) (Olsson & Hahn-Häigerdal, 1996). Therefore, the search of native organisms for sugars (C5 and C6) fermentation processes in different environments, which imposes an adaptive evolution on the cells, could provide a microbial diversity with high potential for the bio-conversion of pentoses and hexoses in ethanol.

2. Methods

2.1 Isolation of bacterial strains

Colombian native bacteria able to transform pentoses were isolated using a selective media with xylose as the sole carbon source. Cultivation was made in liquid medium as previously described by Ronan, *et al.* (2013), with some modifications, as follows: The tubes were sealed with screw cap and covered with parafilm to reduce oxygen diffusion; 15 g of material from each working condition (samples of panela's cane bagasse, sugarcane bagasse, compost piles, bagasse mixtures with compost and soil) were added to 60 ml of culture media, incubated at four different isolation temperatures (30, 37, 45 y 55 °C) and, after 8 days, the resulting culture was sequentially transferred to fresh medium in a ratio of 1:5 (v/v) every 48 h for 12 days, using the same incubation conditions. Each enrichment was subsequently grown into solid culture media.

2.2 Screening of ethanol-producing bacteria

Screening of the axenic isolated cultures for the determination of ethanol production was performed by the double-coupled enzyme assay (oxidase-peroxidase) as previously described by Qi, X. et al. (2011). To determine the selectivity of the enzymatic method, acid and alcohol patterns (formic acid, acetic acid, lactic acid, isopropyl and isobutyl alcohol) were analysed in the assay.

2.3 16S rRNA gene sequence analysis

The enzymatic extraction of genomic DNA from selected isolates was carried out according to Andrews & Patel (1996) and Marmur (1961). Sequencing of DNA was performed by Macrogen Inc. (Korea) using the primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The respective DNA template was added in each reaction at a concentration between 25 and 100 ng/µL. For the classification of isolated ethanol-producing bacteria, the sequences obtained were cleaned according to quality scores using the free software Chromas (http://technelysium.com.au/?page_id=27) the consensus sequences were constructed using the MUSCLE algorithm (Multiple Sequence Comparison by Log-Expectation). The consensus sequences obtained were aligned against the nucleotide database of the National Center for Biotechnology Information (NCBI - http://www.ncbi.nlm.nih.gov/). The sequences with identity percentages higher than 97% were chosen and starting from these were obtained the 16S rRNA gene sequences reported in the bacterial taxonomy database LPSN (List of Prokaryotic names with Standing in Nomenclature- http://www.bacterio.net/).

2.4 Fermentation profiles bacterial isolates

The fermentation profile evaluation was performed in duplicate for each selected isolate in medium supplemented with 1 % xylose or medium supplemented with 2 % glucose and 1 % xylose as carbon source. The isolates were cultured in 1.5 ml microtube in 1 ml liquid medium with 1 % D-xylose as the sole carbon source, for 48 h at 200 rpm, maintaining the original isolation temperature conditions for each strain. Cells biomass was centrifuged at 4 °C and 15,200 rpm for 10 min, washed 2 times with sterile 0.85 % NaCl and the optical density at 600 nm was adjusted to 0.2 +/- 0.03. To determine the fermentation profile, the inoculum (at 10% of the final volume of 5 mL) was added in 5 mL microtube containing fermentation medium, and sealed with parafilm for 48 h at 200 rpm, maintaining the original temperature isolation conditions for each strain. After fermentation, the culture was centrifuged at 4 °C and 15,200 rpm, for 30 min. The liquid culture supernatant was analyzed by HPLC with BioRadAminex® HPX-87H column eluted at 65 °C, 5mM H_2SO_4 as a mobile phase at a flow rate of 0.6 mL/min and a refractive-index detector (Sluiter et al., 2006). Products were identified by comparison of retention times with those of pure substances.

3. Results and Discussion

With the conventional culture methods used, 382 xylose-consuming bacterial isolates were obtained from the different work samples used. More mesophilic isolates were obtained (144 and 110 at temperatures of 37 °C and 30 °C, respectively) than thermophilic isolates (69 and 59 at temperatures of 45 °C and 55 °C, respectively).

To perform the enzymatic method selection of ethanol-producing isolates, the specificity of the screening assay was determined by calculating the selectivity coefficient K of different alcohols and organic acids (Table 1). The values obtained were close to zero, indicating that there is no contribution of the evaluated compounds to the instrumental response (Figure 1).

Table 1: Selectivity coefficients (K) of alcohols and acids determined in the enzymatic method.

Compound	Ethanol	Isobutyl Alcohol	Isopropyl Alcohol	Formic Acid	Lactic Acid	Acetic Acid
Abs 490nm	0.964	0.032	0.064	0.073	0.018	0.000
SD[a]	0.054	0.005	0.012	0.065	0.010	0.000
K[b]	1.000	0.034	0.067	0.076	0.019	0.000

[a] SD= standard deviation (n=3); [b] *K*= Abs compound/Abs ethanol

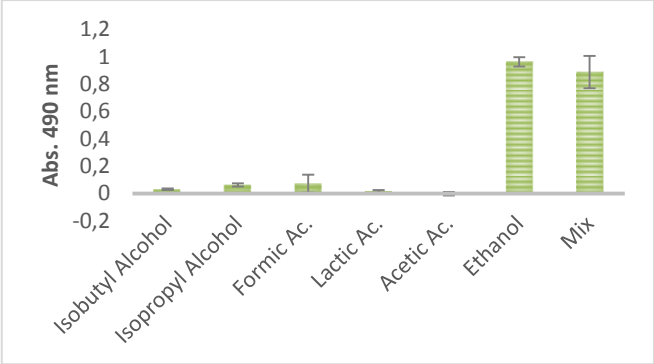

Figure 1. Effect of the alcohols and organic acids on the enzymatic assay. Ethanol, formic acid, lactic acid and acetic acid (2 g L^{-1}); Isobutyl and Isopropyl alcohol (2 % w/w); Mix corresponds to the mixture of all evaluated compounds in the fermentation medium.

In the screening process of ethanol-producing bacteria, only 13.6% of all isolates had a positive response to the enzymatic assay for the presence of ethanol (Figure 2).

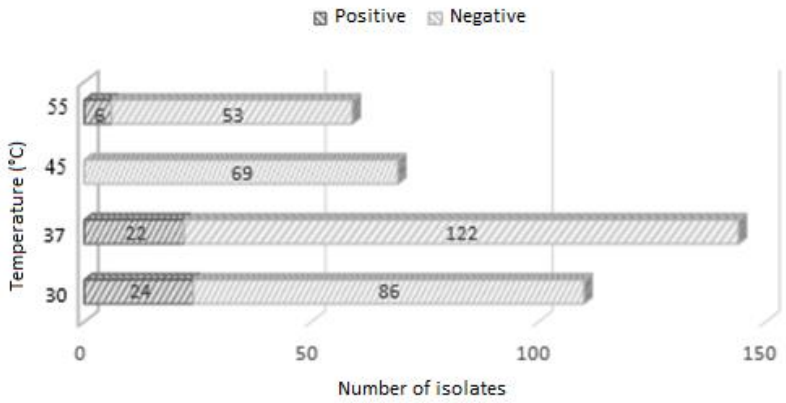

Figure 2. Number of xylose-consuming native isolates recovered at different temperatures, with positive response for ethanol production using the enzymatic assay.

The ethanol-positive thermophilic isolates represent only 1.6% of the total isolates (all of them isolated at 55 °C), whereas the mesophilic isolates positive to the screening assay represent 12% of the total isolates. These results suggest that the isolates with a negative response present the functioning of fermentative metabolic pathways for xylose but ethanol production is not obtained under the evaluated conditions.

Bioinformatics analysis of the sequencing data from the different ethanol-producing bacteria allowed to relate phylogenetically 15 isolates with a percentage of identity higher than 97% (Table 2). According to these, 80% of the isolates belong to the Bacillaceae family, 13.3% to the Enterococcaceae family and 6.7% to the Enterobacteriaceae family. These genera have been previously described in literature for their ethanologenic capacity, altough not necessarily from xylose as sole carbon source (Shariat et al, 1995; Combet-Blanc et al, 1999; Ahmad et al, 2000; Chandel et al, 2011).

Table 2: Reported species with the highest percentage of similarity to ethanologenic isolates.

Strain code	**Gram stain**	**Cell morphology**	**Molecular identification**	
			Microorganism	% Identity[a]
552MC11	Negative	Bacillus	*Bacillus coagulans*	100
552C15	Positive	Bacillus	*Aeribacillus pallidus*	99.3
552MC6	Positive	Bacillus	*Bacillus thermoamylovorans*	99.7
372C18	Positive	Bacillus	*Bacillus galactosidilyticus*	99.8
372C10	Positive	Bacillus	*Bacillus licheniformis*	100
372T15	Positive	Bacillus	*Bacillus sonorensis*	99.4
372C14	Positive	Bacillus	*Bacillus anthracis*	100
302T8	Positive	Bacillus	*Bacillus aryabhattai*	99.7
302T12	Positive	Bacillus	*Lysinibacillus fusiformis*	99.6
302T10	Negative	Bacillus	*Citrobacter amalonaticus*	98.0
302T27	Positive	Bacillus	*Lysinibacillus sphaericus*	99.8
302T30	Positive	Coccus	*Enterococcus mundtii*	99.4
302T4	Positive	Coccus	*Enterococcus faecium*	99.9
302T24	Positive	Bacillus	*Lysinibacillus mangiferihumi*	99.6
302C16	Positive	Bacillus	*Bacillus subtilis subsp. spizizenii*	99.7

[a] %= (1-p)*100; where p is the value of the distance presented in the matrix generated in the MEGA6 program.

The fermentation profiles obtained for the studied isolates (Table 3) correspond to the reports from different studies on the metabolism of xylose in these type of microorganisms, where the formation of by-products is a key factor in the amount of ethanol that can be recovered and depends on the conditions of fermentation; also, the genus of the microorganism can play an important role (McMillan, 1993). In general, in the glucose and xylose mixture, a decrease in the yield of ethanol was observed, with the exception of microorganisms isolated at 55 °C, sugesting that the metabolism of these sugars is through heterofermentative pathways.

Table 3: Fermentation profiles of ethanol-producing isolates in two different media for 48 h.

Strain code	**Media with xylose 1 %**			**Media with xylose 1 % and glucose 2 %**			
	Xylose consumption (g)	**Ethanol yield g g^{-1}**	**%[a]**	**Xylose consumption (g)**	**Glucose consumption (g)**	**Ethanol yield g g^{-1}**	**%[a]**
552MC11	1.90 ± 0.15	0.015	2.94	1.26 ± 0.14	4.31 ± 0.05	0.054	10.55
552C15	1.65 ± 0.04	0.038	7.45	1.45 ± 0.11	2.87 ± 0.05	0.046	9.08
552MC6	3.09 ± 0.07	0.006	1.18	1.74 ± 0.20	3.43 ± 0.03	0.039	7.59
372C18	8.94 ± 0.49	0.112	21.95	0.21 ± 0.02	4.64 ± 0.26	0.000	0.00
372C10	7.35 ± 0.70	0.162	31.85	3.57 ± 0.21	9.49 ± 0.47	0.082	16.11
372T15	8.81 ± 0.50	0.167	32.81	8.28 ± 0.37	18.57 ± 1.25	0.004	0.86
372C14	8.16 ± 0.27	0.174	34.06	1.20 ± 0.06	4.93 ± 0.11	0.050	9.86
302T8	8.68 ± 0.51	0.108	21.20	5.27 ± 0.76	16.51 ± 0.19	0.075	14.75
302T12	1.72 ± 0.06	0.047	9.25	2.98 ± 0.03	14.26 ± 0.17	0.059	11.62
302T10	3.87 ± 0.58	0.129	25.39	1.74 ± 0.01	4.47 ± 0.04	0.010	1.87
302T27	8.78 ± 0.71	0.130	25.39	2.22 ± 0.27	11.61 ± 0.37	0.092	18.12
302T30	8.06 ± 0.19	0.091	17.86	6.87 ± 0.43	18.34 ± 1.53	0.055	10.81
302T4	6.25 ± 0.15	0.098	19.14	0.78 ± 0.04	7.32 ± 0.15	0.016	3.06
302T24	5.89 ± 0.03	0.153	29.92	3.35 ± 0.38	10.96 ± 0.63	0.082	16.05
302C16	6.95 ± 0.07	0.119	23.24	3.02 ± 0.06	10.04 ± 0.59	0.092	18.05

[a]The theoretical yield for the production of ethanol from xylose and glucose is 0.51 g g^{-1} (Olsson and Hahn-Häigerdal, 1996).

The HPLC analysis of the fermentation supernatant with xylose 1% as the only carbon source, showed the formation of several products (Figure 3). In all the chromatograms, the peak marked with number 1 correspond to the amount of xylose remaining after the fermentation process. Figure 3B confirms the superior capacity of isolate 372T15, grown at 37 °C, to metabolize the pentose.
For the 302T10 isolate the chromatogram (Figure 3A) shows the formation of acetic acid (peak 4) and ethanol (peak 5), where ethanol is not its main fermentation product. The fermentation profile for the 372T15 isolated (Figure 3B) revealed the ability to consume xylose and convert it to ethanol as a major product in addition to the formation the organic acids. Isolate 552MC6 exhibited low ethanol production (Figure 3C), corresponding to the low yield percentages obtained for the isolates at this temperature (Table 3). However, it also presented an extensive by-product formation.

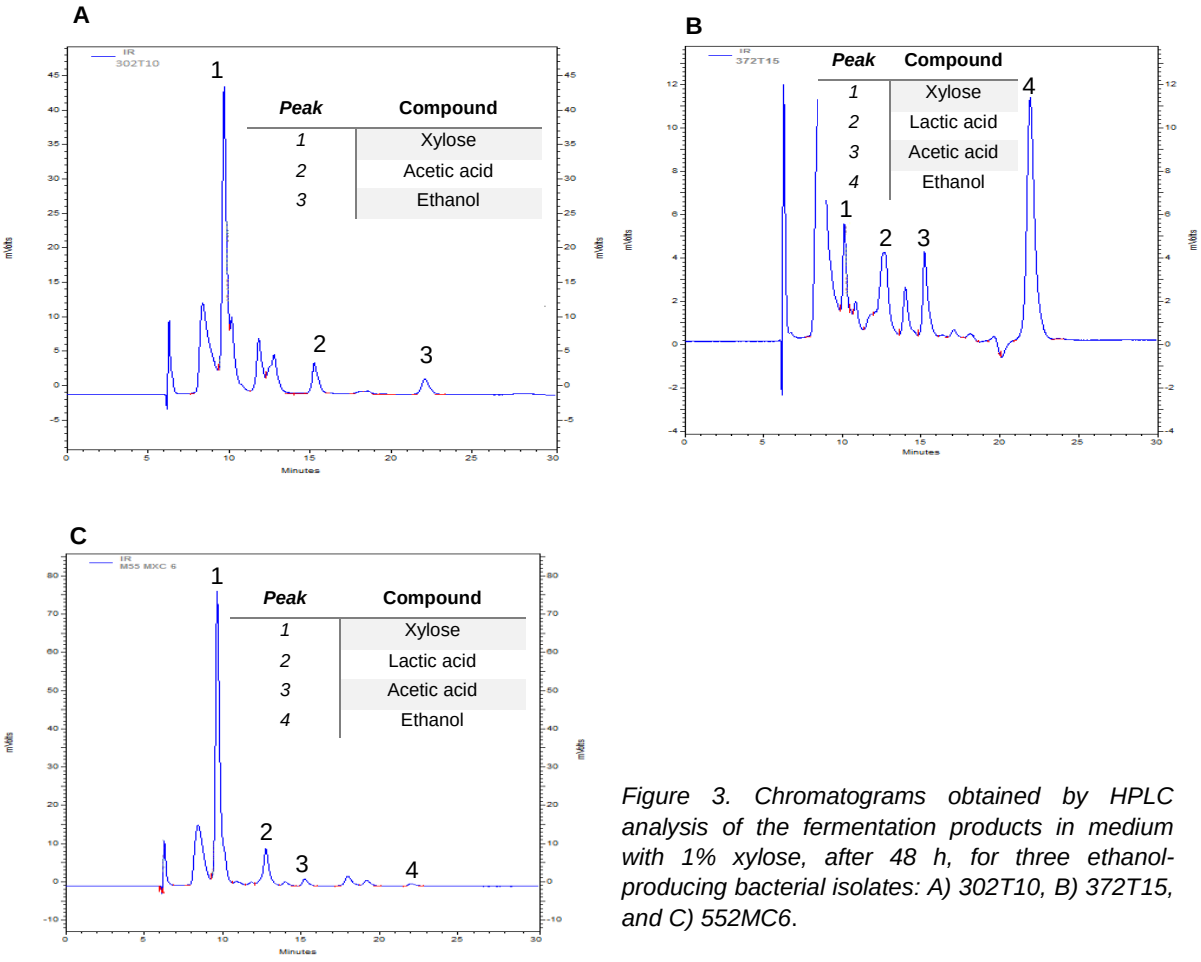

Figure 3. Chromatograms obtained by HPLC analysis of the fermentation products in medium with 1% xylose, after 48 h, for three ethanol-producing bacterial isolates: A) 302T10, B) 372T15, and C) 552MC6.

The fermentation profiles demonstrate that in the isolates the xylose consumption decreased this suggests the action of mechanisms of Carbon Catabolite Repression (CCR) because, when there is a higher concentration of glucose, because it is a readily degraded carbon source, it inhibits xylose uptake mechanisms as a secondary source of energy (Singh et al, 2014). The results showed in the isolates recovered at 30 and 37 °C were obtained major yields percentages in the medium with absence of glucose.

4. Conclusion

Of the total of isolated native bacteria (382) that exhibit the ability to metabolize xylose, a very small number of them presented the biochemical pathway for the transformation of sugar to ethanol. In all isolates studied in this work an heterofermentative process was evidenced, with the presence of organic acids as fermentation by-products

The bacteria studied, despite not exhibiting and achieve high yields for the ethanol production from xylose, offer the feasibility of transforming this important component of the biomass and extend the spectrum of its use because the ability of the bacteria to simultaneously consume xylose and glucose has an advantage for the fermentation process.

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