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RESEARCH ARTICLE

SEMI-SIMULTANOUS SACCHARIFICATION AND FERMENTATION OF *Opuntia ficus-indica* CLADODE FOR BIOETHANOL PRODUCTION USING WILD STRAIN.

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Abstract

Semi-simultaneous saccharification and fermentation processes (SSSF) are capable of improving ethanol production compared with separate hydrolysis and fermentation (SHF) systems because there are reduce of enzymes inhibition due conversion of sugars by yeasts immediately after saccharification.

In this work a wild *Acinetobacter pittii* bacterium and a wild *Kluyveromyces marxianus* yeast were used for SSSF process. The bacterium, isolated from decayed cladodes, had capacity of produce cellulases enzymes, while the yeast, isolated from termite stomach, had fermentative capacity. The SSSF was tested on *Opuntia ficus-indica* cladode flour medium (CFM) with and without agitation (0 and 200 rpm). Ethanol yields (Yp/s) obtained with CFM were 0.43±0.01 and 0.18±0.01 for 0 and 200 rpm, respectively, that represents 84% and 35% of efficiency of SSSF. Ethanol final concentrations reached 11.7±0.02 g/L and 5.80±0.02 g/L for 0 and 200 rpm, respectively. The ability of *A. pittii* for cellulases production combined with *K. marxianus* fermentation capacity in their bests conditions, represented in higher bioethanol production during SSSF.

Introduction:-

Bioethanol was the first biofuel produced from sugar cane and corn as feedstocks. Currently these feedstocks are used for food production; however, great competition for arable land arises. This competition can be avoided by the use of lignocellulosic biomass as feedstock. Transformation of lignocellulosic biomass from municipal solid waste, forestry wastes, and agricultural residues (Cardona Alzate and Sánchez Toro, 2006) to energy fuels and chemicals has garnered considerable research and commercialization interest over the past few decades. Plants that can grow in arid, semi-arid, marginal, degraded, or abandoned lands have the potential to produce bioethanol due to the ability to rapidly absorb and store water in succulent tissues, with the capacity of tolerate large water losses. Crassulacean acid metabolism (CAM) species from genera *Opuntia* are cultivated primarily as commercial fodder in semi-arid regions of Mexico. Nowadays, *Opuntia ficus-indica* represents a surplus of the Mexican agricultural industry. Recently, SIAP (2018) reported a production of 885,435 ton of cladodes in the country; as a result there is an interest in the use of *O. ficus-indica* cladodes for bioethanol production. The efficiency of the conversion of cladodes is limited by the recalcitrance of plant cell wall due to cellulose, hemicellulose and lignin presence. Thus,

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reducing lignin mass fraction and altering cellulose structure are important goals for overcoming this recalcitrance to improve saccharification.

Different treatment methods have been employed to disrupt the cell wall to expose cellulose and hemicellulose fibres for increasing enzyme accessibility (enzymatic hydrolysis or saccharification) or acids/alkalis accessibility (hydrolysis) to biomass and fermentable sugars yields (Haghighi-Mood et al., 2013).

In the literature, several systems for ethanol production at laboratory or pilot scale from biomass have been reported (Cardona Alzate and Sánchez Toro, 2006). Separate hydrolysis and fermentation (SHF) involves two separate steps with two optimal process conditions (OPC), an enzymatic hydrolysis or saccharification (OPC: 50 °C and pH 4.8, for pure and commercial enzymes), and an alcoholic fermentation (OPC: 37-45 °C and pH 5, for yeast) (Cotana et al., 2015); however, the accumulation of glucose and cellobiose during enzymatic hydrolysis inhibit the cellulases, reducing the final efficiency (Margeot et al., 2009). This disadvantage has allowed other process configurations for ethanol production like: simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation, consolidated bioprocessing, and sequential enzymatic saccharification and fermentation (Pérez-Pimienta et al., 2017) called semi-simultaneous saccharification and fermentation (SSSF) (Cotana et al., 2015).
This last scheme (SSSF) consists of a pre-hydrolysis step (short heat treatment for 4-24 h) followed by SSF (Gonçalves et al., 2014) which offers advantages over SHF and SSF to produce higher ethanol concentration, yield, and efficiency (Zhang et al., 2014) due to cellulases inhibition reduction when yeasts consume the free sugars produced in the media (Hahn-Hägerdal et al., 2007).
The main advantage of enzymatic hydrolysis is the use of pure commercial cellulases and xylanases with low energy consumption and generation of fewer fermentation inhibitors. In fact, a synergistic action of three commercial cellulases operating at 40-50 °C and pH 4-5 has been studied (Retamal et al., 1987). In this sense, many microorganisms like fungus, actinomycetes and bacteria are used for cellulases production such as *Bacillus*, *Cellulomonas*, among others (Zaafouri et al., 2016).

Process improvement through sequential optimization of culture condition for enhanced production of xylanases has been reported from wild isolated *Acinetobacter sp* (Lo et al., 2010). Moreover, Purohit et al. (2017) identified and characterized a new bacterial strain *Acinetobacter pittii* MASK25 producing xylanase.

The supernatant from enzymatic hydrolysis containing both hexoses and pentoses, is normally subjected to alcoholic fermentation. Hexoses such as glucose, galactose, and mannose are directly fermented into ethanol by many natural organisms, but pentoses including xylose and arabinose, are fermented into ethanol by a few native strains, usually at relatively low yields. The yeast *Saccharomyces cerevisiae* is the most studied fermenting microorganism for its inherent resistance to low pH, high temperature, and various inhibitors. Other wild type microorganisms used in the fermentation process include *Eschericha coli*, *Zymomonas mobilis*, *Pichia stipites*, *Candida brassicae*, *Kluyveromyces marxianus*, among others (Ballesteros et al., 2004).

On the other hand, the use of thermotolerant microorganisms provides a number of potential advantages in feedstock fermentation. In this context, *Kluyveromyces marxianus* is gaining great significance due to its capability of growing and fermenting at temperatures above 40 °C, close to the optimum temperature of enzymatic hydrolysis, which integrate both, saccharification and fermentation process (Moreno et al., 2013).

In this work the semi-simultaneous saccharification and fermentation with *Acinetobacter pittii* and *Kluyveromyces marxianus* as cellulolytic and fermentative microorganisms, respectively, was evaluated using *Opuntia ficus-indica* cladode as unique carbon source with and without agitation for cellulase and alcohol production.

Material and Methods:-

Raw material

Six month old cladodes of *Opuntia ficus-indica* var Atlixco, were cut in 1 cm^2 cubes and dried at 80 °C for 24 h, then milled to obtain a flour with particle size of 1500 µm. The obtained flour was stored in plastic bags and kept dry for further use.

Microorganisms isolation and growth condition

The isolation and identification of two strains was conducted: a bacteria was isolated from decaying cladodes and a yeast was isolated from termite stomach. Decaying cladodes were liquefied with sterile saline solution (proportion 1:2) while termite stomach was removed from several termites and homogenized with the same solution. The mixtures were serially diluted and an aliquot was spread on solid medium containing minerals and 1% carboximetilcellulose as unique carbon source. Microbial growth and cellulase induction was made at 37 °C for 24 h. Colonies were screened for cellulases activity by Congo test using solid medium with minerals and 1% cladode flour (SCFM). The positive colonies with the maximum zone of clearance were picked and grown in fresh medium (nutritive broth for bacterium and YPD for yeast) and stored at 4 °C. Amplification and sequence analysis of the 16S rRNA gene was performed and microorganisms were identified as *Acinetobacter pittii* and *Kluyveromyces marxianus*. Both microorganism exhibited cellulase activity and only yeast presented fermentative capacity.

Media preparation

For media preparation cladode flour was used in a 20% concentration. A mixed of water (225 ml) and minerals I and II solutions were used. Mineral solution I (12.5 ml): 0.6 % of K_2HPO_4 and mineral solution II (12.5 ml): 1.2 % of NaCl, 1.2% of $(NH_4)_2SO_4$, 0.6 % of KH_2PO_4, 0.12 % of $CaCl_2$ and 0.25 % of $MgSO_4.7H_2O$ (Atlas, 2004). The media was adjusted to pH 5.5 and sterilized by autoclaving (121 °C/15 lb/15 min) considered as a pre hydrolysis.

Semi-simultaneous saccharification and fermentation (SSSF)

A scheme of three steps was perform before the growth kinetics. First step consisted of an adaptation growth in tubes containing 9 ml of 1% cultured medium and 1 ml of strain preserved in glycerol at -20 °C were incubated at 37 °C, 200 rpm during 4 h. The second step was developed in 75 ml of 20% cultured medium using the inoculum, growth at the same conditions during 16 h. Finally, the third step consisted in 250 ml of 20% of medium starting with 20 x 10^6 cell/ml. The SSSF were carried out during 32 h. The bacteria growth during the first 8 h at 37 °C and then the yeast was inoculated with a fixed temperature to 40 °C for 24 h. The SSSF was conducted with and without agitation (0 and 200 rpm).

Analytical procedures

Populations of cell/ml x10^6 were quantified by direct count in microscope of samples dilutions. Observations were made at 40X using Neubauer Brigh-line chamber with 0.1 µl capacity. Reducing free sugars (g/L) were quantified by dinitrosalicylic method at 550 nm (Miller, 1959). Total sugars (g/L) by phenol-sulfuric method by a spectrophotometric determination at 492 nm (Dubois et al., 1956)). Alcohol (g/L) determination was made by potassium dichromate method at 585 nm (Sumbhate et al., 2012). Glucose (g/L) concentration were measured using biochemical analyser (YSI, model 2900D) using a membrane with immobilized enzymes (YSI 2365-glucose oxidase) on the hydrolyzed and fermented samples centrifuged at 4700 rpm for 20 min. The assay of FPase activity was conducted according to IUPAC (Ghose, 1987) method by measuring the reducing sugars in a reaction mixture containing Whatman No. 1 filter paper (1x6 cm≈50 mg) as substrate in 1 ml of 0.05M of sodium citrate buffer (pH 4.8) and 0.5 ml of the crude enzyme at 50 °C after 1 h. Reducing sugars were assayed by the dinitrosalicylic acid method. One unit of cellulase activity (IU/ml) was defined by the formation of 1 µmol of glucose equivalents released per minute under assay condition.

Statistical analysis

The software Statgraphics Centurion XVI was used to analyse population, pH, free reducing sugars, total sugars, alcohol, glucose and FPase activity in the samples obtained from SSSF with and without agitation. The data were analyzed for statistical significance by a one-way analysis of variance (ANOVA) and the multiple range test ($p<0.05$).

Results and Discussion:-

Microorganism growth

In this research, an *Acinetobacter pittii* and *Kluyveromyces marxianus* strains were studied, focused in the production of cellulases activity and alcohol by semi-simultaneous saccharification and fermentation process (SSSF) using *Opuntia ficus-indica* cladode flour as substrate. The maximum growth of *A. pittii* was 725±216 x 10^6 cell/ml at 200 rpm, in both agitation condition maximum growth was achieved at 6 h. After this time, an important decrease in population was observed and a stationary phase was presented with the inoculation of the yeast. *K. marxianus* had an exponential growth reaching a maximum growth of 156±20x10^6 cell/ml observed at 0 rpm (Fig. 1), without

statistical differences between SSSF at 0 rpm and at 200 rpm for both strains. Yeast population was lower than bacterial population with similar behaviour in both conditions.

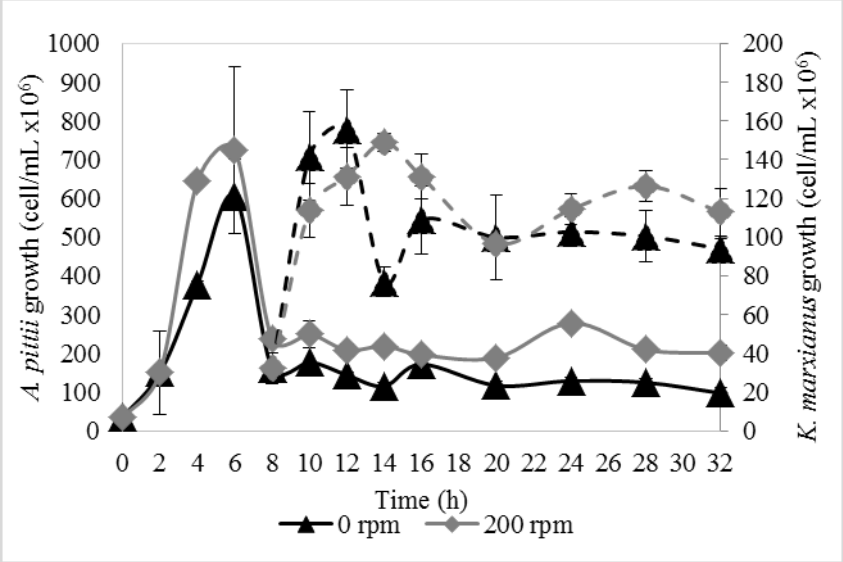

Fig. 1:-Growth curves of Acinetobacter pittii (solid lines) and Kluyveromyces marxianus (dashed lines) in cladode flour medium (20%)

Figure 2 shows the pH change in the cultured media during SSSF fermentation (first 8 h with bacterium and then with both microorganisms). An increase of 0.2 units of pH was observed during the first 12 h at 0 rpm while at 200 rpm an opposite behaviour was observed since there was a decrease of 0.3 units during first 4 h. According to the ANOVA there was a significant effect on pH due to the agitation. The gradual increase of pH in the medium without agitation allowed ethanol production. Singh and Bishnoi (2013) reported a similar pH effect for *S. cerevisiae* on wheat straw hydrolysates, observing a higher ethanol production at pH of 5.5.

Morales et al. (2015) have mentioned a negative effect on the production of ethanol when agitation is present, since the increase of acids (such as acetic acid) in the medium can modify the conformation of proteins in the plasma membrane and thus impact the permeability of certain ions and other metabolites. In the present work, the decrease of pH was observed when agitation was used, the small change may be related to the lower production of ethanol under this conditions. The highest ethanol concentration (Fig. 3) was obtained without agitation at a pH of 5.6, while sugar consumption was equal in both conditions (Fig. 3).It is important to maintain a constant intracellular pH (up to 6 units) because there are many enzymes functioning within the yeast cell during growth and metabolism.

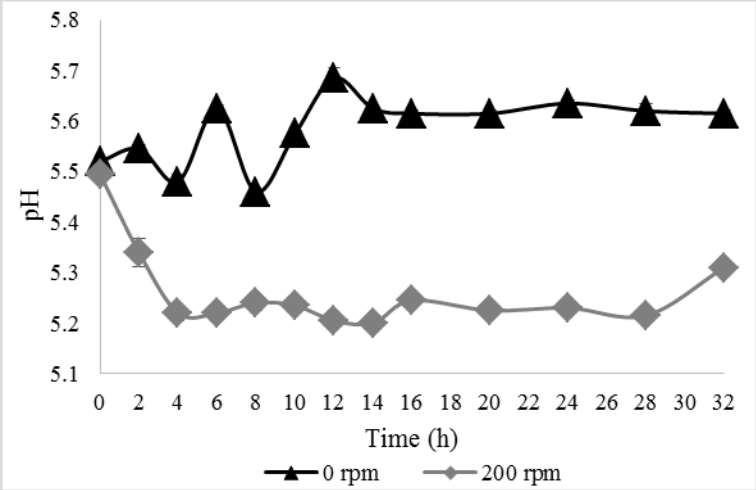

Fig 2:-pH change in cultured medium during SSSF

Semi-simultaneous saccharification and fermentation

As shown in Figure 3, in both processes (0 and 200 rpm) the bacterium *A. pittii* was capable to assimilate the free sugars present in cladode medium for growth after the first 4 h. At this time bacterium used that carbon source first, for enzymes production and then, hydrolyzed the polysaccharides for sugars release. The maximum total sugars (total sugars = mono-, di-, oligo-, and polysaccharides) was obtained at 0 rpm reaching 26±1.12 g/L at 8 h and then a decrease was observed due the use of that sugars in the medium by yeast for alcohol production with a maximum at 16 h (just 8 h after yeast inoculation) being 11.07±0.02 g/L the final alcohol concentration. After this maximum appeared, alcohol concentration decreased maybe due to consume by the microorganisms as carbon source for maintenance or for organic acids formation. The alcohol concentration obtained was less than Kuloyo et al. (2014) obtained (25 g/L), but the hydrolysis conditions were conducted with sulfuric acid as the hydrolysing agent. The yields, fermentation efficiency and productivities obtained for 0 and 200 rpm are presented in Table 1. Ballesteros et al. (2004) reported yields and efficiency of 0.32 and 61%, respectively for the simultaneous saccharification and fermentation (SSF) of wheat straw using *K. marxianus* CECT 10875 and a substrate concentration of 10%. The feasibility of using 20% substrate concentration in SSSF with wild *K. marxianus* is considered relevant, since earlier studies have reported the limiting effect of high substrate concentrations due to the difficulties of the agitation or high ethanol inhibiting concentrations (Mohagheghi et al. 1992). Nevertheless, the cellulose content of cladode substrate used was not determined. Unlike the separate hydrolysis and fermentation (SHF) in the semi-simultaneous process the inhibition by product released decreases since glucose and other simple sugars (fructose, xylose, among others) are consumed by the yeast as soon as it is formed by bacterium enzymes. At 200 rpm concentrations of total sugars and final alcohol were lower than obtained at 0 rpm. According to the ANOVA (Fig. 4), a significant effect was found in alcohol production, yield and efficiency due to agitation, being 0 rpm the best condition for alcohol production and the kinetics parameters.

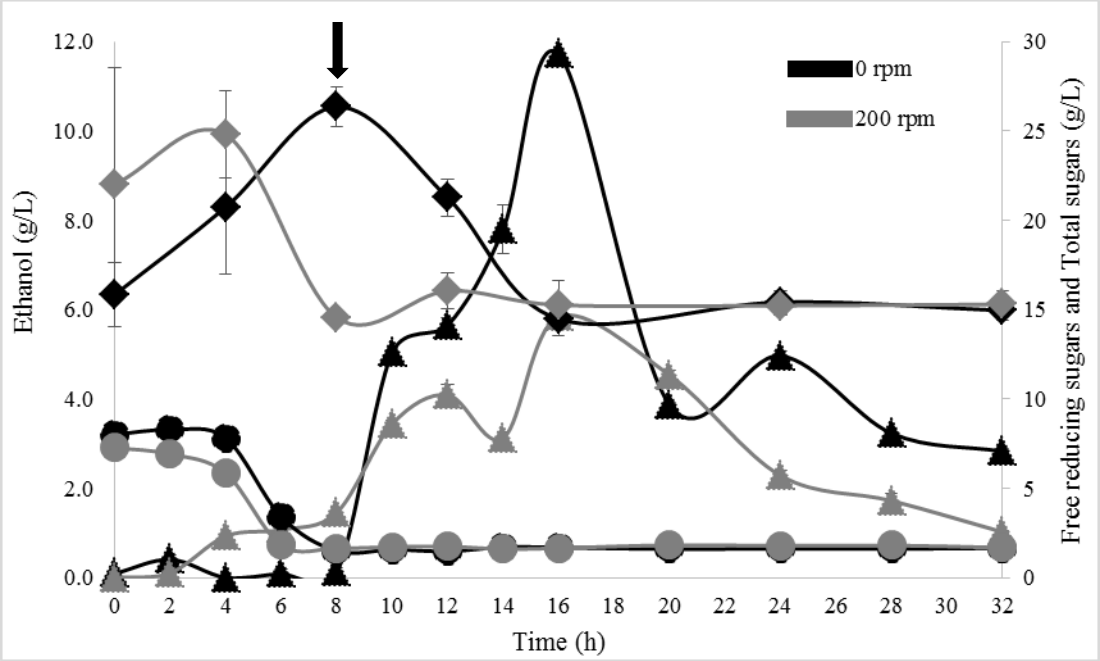

Fig 3:-Total sugars production (diamond), free reducing sugars consumption (circle) and ethanol production (triangle) during 32 hours of semi-simultaneous saccharification and fermentation on cladode flour medium. Yeast inoculation (black arrow)

Table 1:-Ethanol production and kinetics parameters

SSSF (rpm)	Ethanol (g/L)	Y p/s[a] (dimensionless)	Efficiency (%)[b]	Productivity (g/Lh)
0	11.07±0.02	0.43±0.01	84	0.73
200	5.80±0.02	0.18±0.01	35	0.37

Substrate concentration (20%)

[a] Yield product/substrate, g ethanol/g FRS (expressed as potential glucose) in cladode flour medium enzymatically hydrolyzed

[b] As percentage of the maximum theoretical ethanol yield (0.51 g ethanol/g glucose)

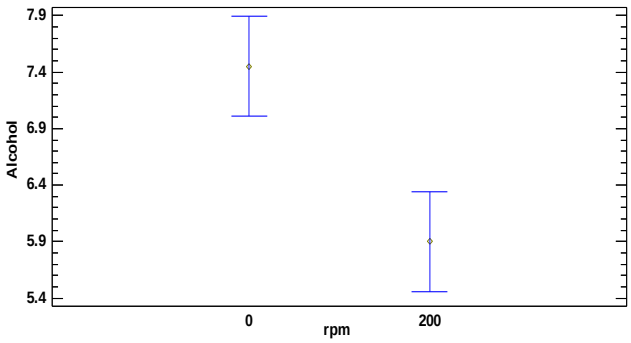

Fig 4:-Means plot for alcohol production

Total cellulases (FPase) production for each condition of the process is showed in Figure 5. The maximum activity was 0.49±0.005 U/ml and was observed at 0 rpm and 10 h, this correspond with the initial stationary phase, 2 h after inoculation, when total sugars were used for ethanol production. According to Ekperigin (2007) the maximum enzyme activity of *Acinetobacter anitratus* strain in carboximetilcellulose was 0.48 IU/ml. The difference of activity with *Acinetobacter pittii* lies in the microorganism specie and type of carbon source.

At 200 rpm the maximum activity was 0.39±0.001 U/ml, recorded at 10 h similar to the unagitated medium. Enzymatic hydrolysis processes (EHP) are of major interest because enzymes catalyze only specific reactions; therefore, unlike acid hydrolysis, there are no side reactions or by-products so EHP can potentially be conducted with high yields. According to the ANOVA, the agitation had a significant effect on the activity of total cellulases with differences between processes at 0 rpm and 200 rpm.

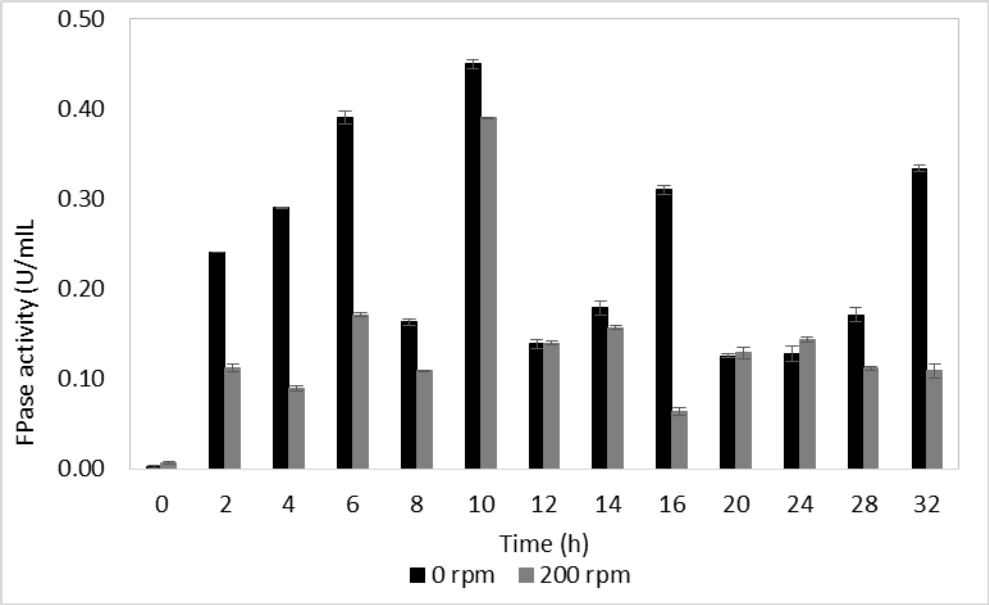

Fig 5*:-*FPase activity (total cellulase) of supernatant

Conclusions:-

Based on experimental results of this study, it is suggested that the use of semi-simultaneous saccharification and fermentation (SSSF) by first growing wild *Acinetobacter pittii* at 37 °C and second growing of thermotolerant wild *Kluyveromyces marxianus* strain at 40 °C represents a novel and efficient process, with high ethanol production using *Opuntia ficus-indica* cladode flour as novel carbon source. *K. marxianus* can metabolize the sugars obtained by the cellulases excreted by *A. pittii*. *A. pittii* had high growth in comparison with *K. marxianus* during SSSF. Saccharification of cladode medium and sugars fermentation using SSSF lead to high cellulase activity reaching a good alcohol concentration in an unagitated process.

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