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Innovative of Second Generation Ethanol Production from Biomass Crops by *Pichia Stipitis*

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The production of ethanol from lignocellulosic material, as reported by Kumar (2009), consists of mainly five different steps, namely pretreatment, (enzymatic) hydrolysis, fermentation, product separation and post-treatment of the liquid fraction. The pretreatment is necessary to improve the rate of production and the total yield of monomeric sugars in the hydrolysis step. The produced monomeric hexoses (six carbon sugars) can be fermented to ethanol quite easily, while the fermentation of pentoses (five carbon sugars) is only made by a few strains. It's been choosen a strain of *Pichia stipitis* wich is able to convert hexoses and pentoses to ethanol.
Preliminary tests with *P. stipitis* and synthetic broth containing glucose and xylose confirmed the utility of *P. stipitis* to produce ethanol by hexoses and pentoses.. Pretreatment (acid and alkali), hydrolysis with enzymes and fermentation with *P. stipitis* allowed to produce ethanol by using mulberry as biomass.

1. Introduction

The lignocellulosic material is widely available but it is yet used for the production of bioethanol. Many factors, like lignin content, crystallinity of cellulose, and particle size, limit the digestibility of the hemicelluloses and cellulose present in the lignocellulosic biomass as reported by Hendriks (2009). The pretreatments are necessary to improve the digestibility of the lignocellulosic biomass. Each pretreatment has its own effect(s) on the cellulose, hemicelluloses and lignin; the three main components of lignocellulosic biomass. Cellulose exists of d-glucose subunits, linked by b-1,4 glycosidic bonds. Hemicellulose is a complex carbohydrate structure that consists of different polymers like pentoses (like xylose and arabinose), hexoses (like mannose, glucose and galactose), and sugar acids. The lignin is, after cellulose and hemicellulose, one of the most abundant polymers in nature and it is present in the cellular wall. It is an amorphous heteropolymer consisting of three different phenylpropane units (p-coumaryl, coniferyl and sinapyl alcohol) that are held together by different kind of linkages.
The production of ethanol from lignocellulosic material consists in mainly five different steps, as reported by Kumar (2009), namely pretreatment, (enzymatic) hydrolysis, fermentation, product separation and post-treatment of the liquid fraction. The pretreatment is necessary to improve the rate of production and the total yield of monomeric sugars in the hydrolysis step. As reported by Dogaris (2013) several reports and reviews have been published on production of ethanol fermentation by microorganisms, and several bacteria, yeasts, and fungi have been reportedly used for the production of ethanol. The monomeric hexoses (six carbon sugars) produced can be fermented to ethanol quite easily, as reported by Lin (2006), while the fermentation of pentoses (five carbon sugars) is only made by a few strains. It's been choosen a strain of *P. stipitis* wich is able to convert hexoses and pentoses to ethanol as reported by Chang (2013), Xavier (2010) and Huang (2009).
It's been studied a process to convert mulberry lignocellulosic stem in ethanol by means pretreatment, hydrolysis and fermentation with *P. stipitis*.

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2. Methods

2.1 Microorganism and maintenance

Pichia stipitis used in this study has been taken from DSMZ collection (3651). The media used for growth, maintenance and inoculum preparation were YM broth and YM agar containing (g/l): yeast extract 3; malt extract 3; peptone from soybeans 5; glucose 10; agar 15; pH 5.6. The media has been sterilized by autoclaving at 394K for 15 min.

It's been used, in the preliminary tests, the YM broth medium with malt extract (YM) and without malt extract (YM-EM) and with double glucose concentration to evaluate the microorganism growth at different conditions. Tests were also performed under the same conditions by replacing glucose with xylose and also with glucose/xylose mixture.

2.2 Treatments

H_2SO_4 2% was added to the crushed (18 mash) and dried (378K) sample, with ratio of 5% w/v (about 5 g of dry biomass in 100 ml of acid in a bottle of 250 ml). The sample was incubated in shaker at 333K for 24h with agitation of 130rpm. This step has the purpose of removing the hemicellulose as reported by Curreli (2002). After the incubation, the hydrolyzate sample was filtered and the residue was washed with distilled water to loss the acidity. The residue was dried overnight at 378K and the filtrate was preserved at 277K.

NaOH 1% was added to dry residue, always using a biomass concentration of 5% w/v. The sample was incubated in shaker at 313K for 24h with agitation of 130rpm and, at the end, H_2O_2 was added up to 1% in the solution and was brought the temperature at 298K. The sample has been incubated again in the dark conditions to avoid photo-decomposition of hydrogen peroxide. This step has the purpose to remove the residual lignin and hemicellulose of the previous step as reported by Curreli (1997). The residue was filtered and washed with acetic acid 0.1M to break down the alkalinity and then with deionized water to remove the acidity. The residue was dried overnight at 378K; the filtrate was preserved at 277K. At the end, a few quantity of dry residue was taken to determine the fiber composition by Van Soest method as reported by Zhao (2012).

All filtrates from previous treatments were added with: 4.5 g/l KH_2PO_4; 2 g /l $(NH_4)_2SO_4$; 0.5 g/l NaCl; 1 g/l yeast extract; 100 µl/l $CaCl_2$ 1M; 1 ml/ $MgSO_4$ 1M; pH 5 ± 0.2; and then with dry residue. After the solution was sterilized at 121 ° C for 0,25h in a 0.5L Erlenmeyer flask. At the sterilized solution were added, in sterility conditions, the enzymes at the dose of 20 FPU for gram of cellulose (cellulase from Tricoderma reesei, Celluclast 1.5L, Sigma C2730) and 30 CBU for gram of cellulose (cellobiase from Aspergillus niger, Novozyme 188, Sigma C6105). The enzymatic reaction temperature was kept constant at 313K for 72h at 110rpm in an incubation shaker. A single sample was taken at the end and stored at 253K until analysis by HPLC.

At the end of the 72h of hydrolysis, 0.3ml of 6M NaOH was added to form a phosphate buffer at pH 6. The solution, fermentation medium, was inoculated with 10% v/v inoculums (18h culture, $1x10^{11}$ cells/L of *P. stipitis*). The fermentation temperature was kept constant at 303K 110rpm in an incubation shaker. During fermentation, every two hours, a sample was took to monitor the consumption of sugar, the ethanol production and the pH trend.

3. Analytical methods

3.1 Raw material characterization

Total solids were determined in according to Reports ISTISAN 96/34 pag. 7.

Cellulose, hemicellulose and lignin contents were determined by Van Soest method.

Insoluble fiber and soluble fiber were determined based on AOAC Method 991.43 by Megazyme

Sugars were determined individually by HPLC method using a Perkin Elmer Flexar Analytical HPLC (column HPX-87P Biorad; mobile phase ultrapure water; flow 0,6 ml/min; temperature 358K; run time 35 min; IR detector).

3.2 Sugar and ethanol determination

Glucose, xylose and ethanol was estimated by HPLC method using a Perkin Elmer Flexar Analytical HPLC (column HPX-87H Biorad; mobile phase 5 mM; H_2SO_4; flow 0,6 ml/min; temperature 338K; run time 25 min; IR detector).

4. Results and discussion

Before to ferment biomass hydrolyzates were carried out preliminary tests with synthetic media to test the ability of *P. stipitis* to metabolize pentoses and hexoses to produce ethanol.

4.1 Preliminary tests

In *Table 1* are reported the tests' operative conditions carried out.

Table 1: Operative conditions for preliminary tests

Test	Medium	Glucose (kg/m^3)	Xylose (kg/m^3)	Temperature (K)
1	YM	10	0	303
2	YM – EM	10	0	303
3	YM – EM	20	0	303
4	YM – EM	0	10	303
5	YM – EM	0	20	303
6	YM – EM	10	10	303

In Figure 1 is reported the glucose trend for tests carried out to verify the *P. stipitis* ability to consume glucose. In 10 hours the strain is able to consume all glucose (17 kg/m^3). In Figure 2 is reported the ethanol production for the same tests to verify the *P. stipitis* ability to produce ethanol by glucose. With media more rich in glucose we have obtained a greater amount of ethanol produced.

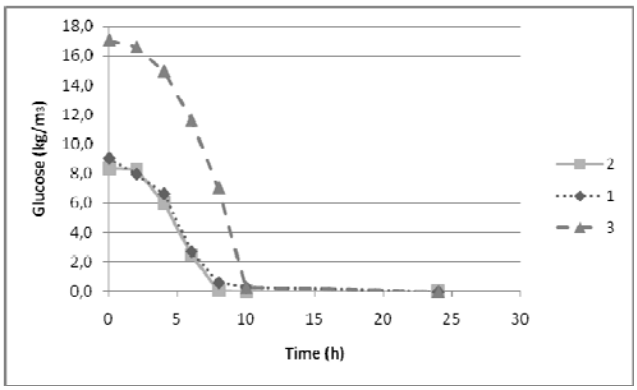

Figure 1: Glucose vs time for preliminary tests with glucose

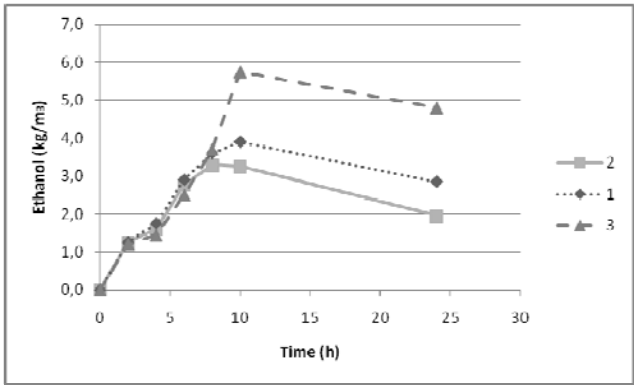

Figure 2: Ethanol vs time for preliminary tests with glucose

In Figure 3 is reported the xylose trend for tests carried out to verify the *P. stipitis* ability to consume xylose. In 24 hours the strain is able to consume 10 kg/m^3 of xylose, to consume 17 kg/m^3 di xylose are necessary 30 hours. Comparing the glucose (Figure 1) and the xylose (Figure 3) trend it can see that there is a lag phase of 4 hours for xylose consumption.

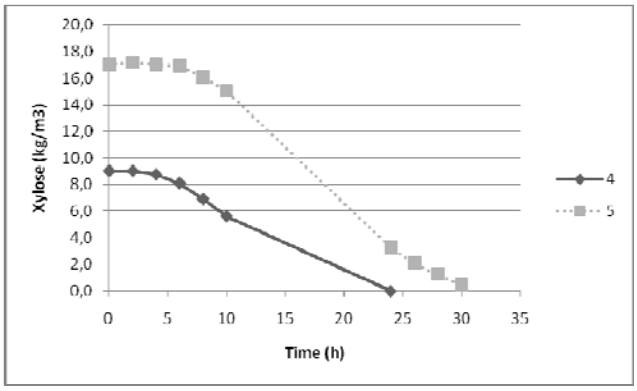

Figure 3: Xylose vs time for preliminary tests with xylose

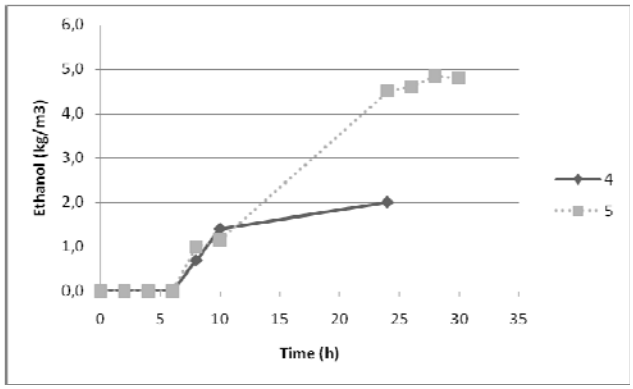

Figure 4: Ethanol vs time for preliminary tests with xylose

In Figure 4 is reported the ethanol production for test carried out to verify the P. stipitis ability to produce ethanol by xylose. With media more rich in xylose we have obtained a greater amount of ethanol produced.
In Figure 5 is reported the glucose and xylose trends for test carried out to verify the *P. stipitis* ability to consume glucose/xylose mixture. In 8 hours the strain is able to consume 10 kg/m^3 of glucose, in 24 h is consumed also all xylose.

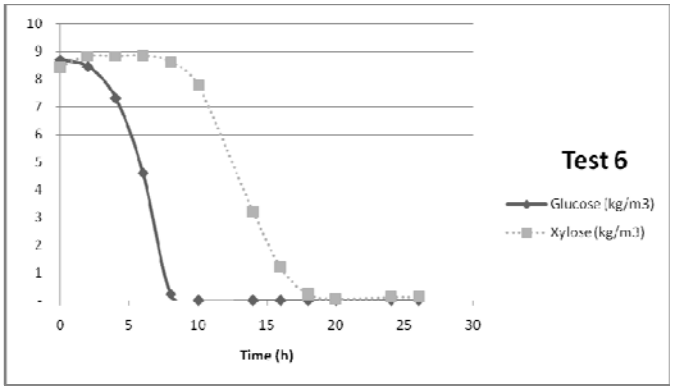

Figure 5: Glucose and xylose vs time for test 6

In Figure 6 is reported ethanol production for test carried out to verify the P. stipitis ability to produce ethanol by glucose/xylose mixture.

The last test was carried out from mulberry lignocellulosic stem with treatment and fermentation. In Table 2 is reported the chemical composition of mulberry lignocellulosic stem used in the test.

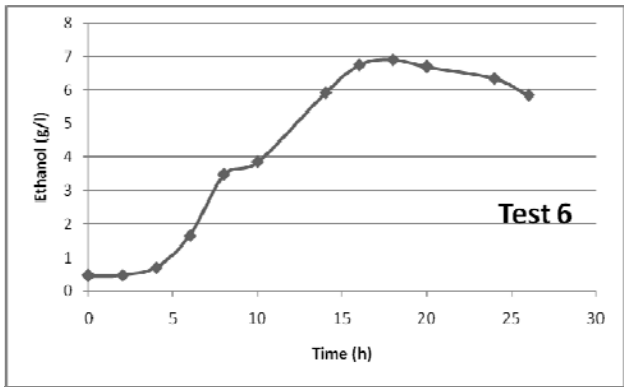

Figure 6: Ethanol vs time for test 6

Table 2: Chemical composition of mulberry lignocellulosic stem used in the test. Values expressed in Kg/Kg

Total solids	Fructose	Glucose	Sucrose	Xylose	Cellulose	Hemycellulose	Lignin	Soluble fiber	Insoluble fiber	Protein
0,910	0,017	0,014	0,014	-	0,137	0,035	0,056	0,045	0,378	0,215

From 0.005 kg of mulberry we obtained 0,17L of fermentation medium with 1,24 kg/m^3 of glucose and 0,74 kg/m^3 of xylose. To activate xylose consumption is necessary to enhance xylose concentration as reported by Agbobo (2006), but also to obtain more ethanol, were added at the medium glucose and xylose of up 5 kg/m^3 of each.

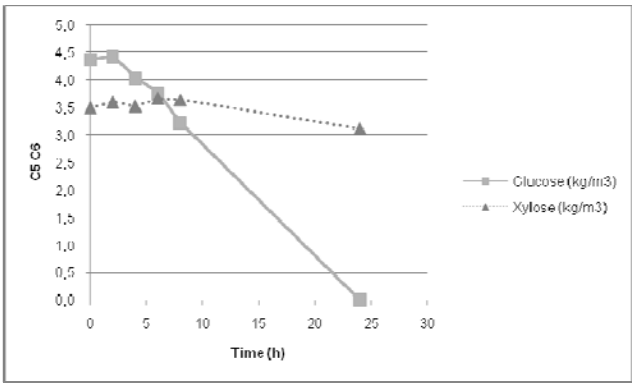

Figure 7: Glucose and xylose vs time for test from mulberry lignocellulosic stem treatments

In Figure 7 is reported the glucose and xylose trends for test carried out to verify the *P. stipitis* ability to consume glucose/xylose mixture from mulberry lignocellulosic stem treatments. In 24h the strain is able to consume all glucose but only the 11% of xylose. The ethanol production is 2,6 kg/m^3.
In Figure 8 is reported the ethanol trend for test carried out to verify the *P. stipitis* ability to produce ethanol from glucose/xylose mixture from mulberry treatments. In 24h the strain is able to consume all glucose but only the 11% of xylose. The ethanol production is 2,6 kg/m^3.

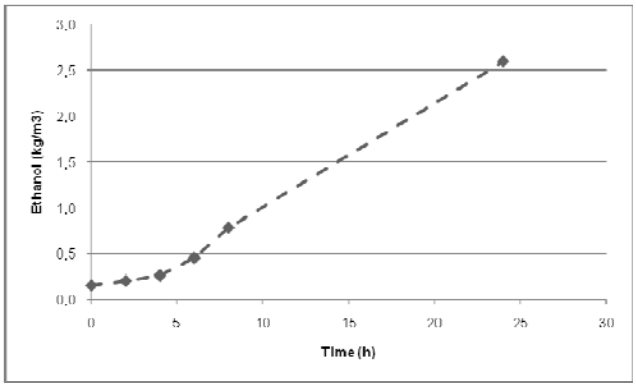

Figure 8: Ethanol vs time for test from mulberry lignocellulosic stem treatments

5. Conclusions

The selected *P. stipitis* strain is able to convert glucose, xylose and glucose/xylose mixture in ethanol.
The LAG phase for xylose consumption lasts 4-5 hours and the glucose one did not show LAG phase.
The average production of ethanol of glucose fermentation is equal to 0.39 kg ethanol/kg glucose (test 1, 2, 3). The average yield of ethanol of xylose fermentation is equal to 0.25 kg ethanol/kg xylose (test 4 and 5).
The chemical/enzymatic treatments of mulberry lignocellulosic stem is efficient to improve total yield of monomeric sugars, C5 and C6, in the liquid phase. 1 kg of mulberry contains 0.27 kg of carbohydrates; the 25% of these are transferred in the liquid phase by treatments (considering only glucose and xylose).
The yield of ethanol from treatments of mulberry lignocellulosic stem is equal to 0.55 kg ethanol/kg sugars. This yield is greater than yield obtained in synthetic medium although xylose has not been entirely consumed.

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