Management of citric solid wastes to produce bioethanol

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ABSTRACT

The increase of the cost of oil and declining oil reserves has led to the search of alternative sources of renewable energy, such as the use of orange wastes.
The aim of this work was the optimization of ethanol production trough a biologic pretreatment (BP) and a simultaneous enzymatic saccharification and fermentation process (SSF) of orange wastes. The BP was made in liquid cultures with *T.harzianum, T.reesei, P.ostreatus,* and *A.niger*. The SSF was made with *T.harzianum*'s cellulase and *K.marxianus* as a fermenting microorganism. We determine the cellulase activity and found that *T.harzianum* (42.23 U/ml) had the best results. Total reducing sugars and the alcohol contents were analyzed with gas chromatrography. The results show that the celluase addition increase the total reducing sugars comparing to biological pretreatment. We obtain 2-3% of ethanol production. After the scale laboratory optimization we will test these processes in 1L fermenter.

Keywords: bioethanol, simultaneous saccharification and fermentation, citrus waste.

1 INTRODUCTION

The progressive depletion of the energetic resources, based principally on unrenewable fuels is increased for the rapidly energetic consumption. The use of renewable energetic resources may solve these problems. The renewable energy is obtained from inexhaustible natural resources throw regeneration. There are two categories: not pollutant (solar, wind, hydraulics and geothermic energy) and those that produce carbon dioxide from organic resources or biomass and they can use as fuels: bioethanol or biogas. The bio-fuels are derivated from vegetal biomass, and they have similar characteristic to the fossils fuels. The environmental and economic advantages are reducing the fossil fuel dependence, improving the combustion, easy production and storage and less contamination because they do not contain sulphur, the principal effector of acid rain, and they do not contribute to the green effect.
However, some studies show that the production is more expensive than the conventional fuels, and that the energy balance is negative.

Bio-ethanol is produce from corn, sorghum, potatoes, wheat, sugar cane, and biomass. Theoretically, we could get ethanol from all organic biomass that contain sugars and starch, but the biomass availability determines the commercial viability (http://www.biodieselspain.com, 2007). The agro industries produce millions of residues annually. These residues are dry, press and they sell as animal aliment to a low cost or they threw away.
This biomass (for example, oranges) could be used as substrate for bioethanol production. One ton of oranges produce between 0.40 to 0.50 ton of juice and 0.50 to 0.60 ton of residues (Fito P., 2007).
The advantages of the use of these residues are: the revalorization of the subproduct and the excedents, the decrease in the dependence in subsidies to make profitable the product, and the reduce in the problematic of the burning of agroindustrial residues (Fito P., 2007).
The most common process to produce bioethanol from these susbtrates consists on the fermentation process.
The simultaneous saccharification and fermentation (SSF) is an attractive process to produce ethanol from lignocelluloses and from the residues of the agro industrial process (Ballesteros, 2004).
The benefits of doing the two processes simultaneously are the reduction in the hydrolysis inhibition for the final products and the low costs of the process.
There are some difficulties to optimize the temperature and the pH for the process, and determine the way to recycle the fermentation microorganism and the enzyme (Olofsson, 2008). Some SSF need a pretreatment before to alter the lignocellulosic matrix and to increase the enzymatic hydrolysis of the cellulose, minimizing the final inhibitory products. There are specialized enzymes denominated cellulolytic enzymes that cut the β-1-4-glicosidic bonds.
The cellulolytic enzymes divide in three sub types depend on their activity: endoglucanases, exoglucanases and and β-glucosidases. There are many microorganisms that can produce this type of enzymatic complex, filamentous fungus, and some anaerobic microorganism among others (Olofsson, 2008).
However, only a few produces extracellular enzyme able to hydrolyze cellulose, this is the case of *Trichoderma reesei*, wich produce endo-β-1.4-glucanases and exo-β-1.4-glucanases (Dashtban, 2009). This microorganism is one of the most studied and characterized as cellulolytic

microorganism (Seiboth et al., 1997; Teeri et al., 1998).
The aim of this work is the optimization of the SSF process using oranges residues as substrate and *Kluyveromyces marxianus* as fermentation microorganism.
Before the SSF we realize a biologic pretreatment of the residues with *Trichoderma harzianum*, *Trichoderma reesei*, *Pleurotus ostreatus*, y *Aspergillus niger*.

2 MATERIALS AND METHODS

2.1 Screening of fungal strains for pretreatment.

Waste of orange (peal and pulp) 100 g, Potato Dextrose media (PDA), 200ml and 2.4% of inoculum were placed in a 500ml flask. Liquid cultures of *T. harzianum*, *T. reesei*, *P. ostreatus*, and *A. niger* in Potato Dextrose Broth (PDB) were used as inoculum. The flask were incubated at 32ºC for 72 hours. After this time, total reducing sugar were determined by the method of 3,5-dinitrosalicylic acid (DNS) at 570nm.

2.2 Propagation of *T. harzianum* y *P. ostreatus.*

T. harzianum and *P. ostreatus* were inoculated in 100ml of PDB and 1g of orange waste. The flask were incubated at 32ºC for 5 days and the pellets were removed and scattered in vortex.

2.3 Optimization of pretreatment using *T. harzianum* and *P. ostreatus* consortium – Study of multiple variables.

The variables used in this assay where: inoculum concentration, temperature and pH. Two levels of each variable were used (Table 1).

For pretreatmente optimization were used 100g of orange waste as substrate. The waste was ground, dried and sterilized at 121ºC for 30 minutes. Then, their were inoculated with *T. harzianum* and *P. ostreatus* and incubated for 10 days under conditions that are show in Table 1. Finally, 10ml of destilled water was added at 10g of sample. The mixture was stirred for 10 minutes at 30ºC and then the extraction of fermentable sugars was done by the DNS method.

Assay	[Inoculum]	pH	Temp. (ºC)
1	1g *T. harzianum* + 2g *P. ostreatus*	5	15
2	1g *T. harzianum* + 2g *P. ostreatus*	5	30
3	1g *T. harzianum* + 2g *P. ostreatus*	7	15
4	1g *T. harzianum* + 2g *P. ostreatus*	7	30
5	2g *T. harzianum* + 1g *P. ostreatus*	5	15
6	2g *T. harzianum* + 1g *P. ostreatus*	5	30
7	2g *T. harzianum* + 1g *P. ostreatus*	7	15
8	2g *T. harzianum* + 1g *P. ostreatus*	7	30

Table 1. Conditions of the different eight assay obtained by combining the differents variables.

2.4 SSF using *Kluyveromyces marxianus*

The differents SSF were carried out in parallel using *K. marxianus* (ATCC 10022) growing in the ATCC recommended media. 50 gs of the pretreatment substrate was inoculated with 25 U of *T.harzianum* cellulases/ ml, and the other 50 gs was inoculated with 25 U of *T.harzianum* cellulases/ ml and 30ml of pellets of *K. marxianus*. The flasks were incubated at 50ºC for 72 hours and then the content of ethanol was analized by gas cromatography in Fermentables Beverage Sector (LATU). Finally, the content of reducing sugars was determinated by DNS method.

2.5 Scale up of pretreatment

For the scale up, 20kg of orange waste were ground and dried and then were treated according to the optimum conditions (Table 1). After 10 days of incubation, reducing sugars were extracted and determined by DNS method.

2.6 Scale up of SSF

The scale up experiment was carried out in 1 litre bioreactor (Bioflo II, New Brunswick Scientific Co. Inc., New Jersey, USA). 100g of pretreatment orange wastes were place in the bioreactor and 800ml of distilled water were added. The operating system condition were: Temp. 48 ± 0,1 ºC and pH of 4.5 ± 0,01. The pH was adjusted to 4.5 with 5N NaOH solution. The agitation was maintained at 120 ± 1 rpm. The enzyme concentration used for the experiment was 0.56gs (40.000U/g) of Powder chinese celullases (Sunson Industry Group Co., Ltd.) and 60ml ($1.4.10^8$cells/mL) of ATCC media with *K. marxianus*. Reducing sugars were extracted and determined by DNS method.

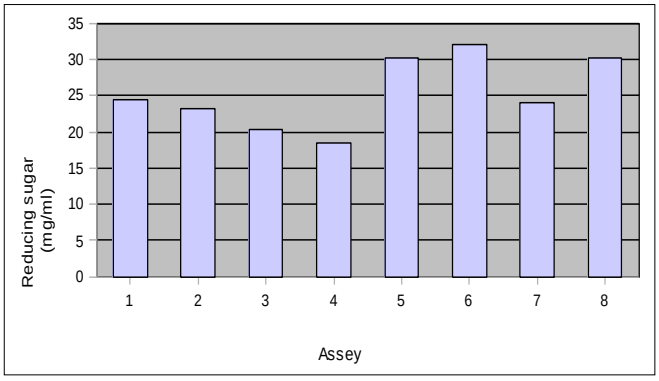
3 RESULTS AND DISCUSSION

3.1 Determination of reducing sugars after biological pretreatment

The screening with the different fungi shows that reducing sugars between the two species of *Trichoderma* do not differ (*T. reesei*: 42.32 mg/mL and *T. harzianum*: 42.35 mg/mL). The reducing sugars obtained from the pretreatment with *P. ostreatus* (23.38 mg/mL), are half than the values obtained with *T. harzianum* y *T. reesei*.
Finally, we may conclude that *A. niger* (16.01mg/mL) did not considerably increase the amount of sugar.
We choose the two genera that showed better results: T. harzianum- P. ostreatus. We propose the use of a consortium T. harzianum- P. ostreatus for optimization of the biological pretreatment as a new strategy.

3.2 Optimization of pretreatment

As we can see in Graphic 1, the assays which used 2g of *T. harzianum*, 1g of *P. ostreatus* at 15 ºC and 30ºC and pH of 5 for 10 days produced higher concentration of reducing sugars. According to the results, this type of pretreatment has good results in a wide range of temperature.



Graphic 1. Results obteined in biological pretreatment optimization

3.3 SSF using *K. marxianus* - Reducing sugar and ethanol determination

We evaluated the reducing sugar consumption by analyzing samples before and after the addition of cellulase of *T. harzianum* and *K. marxianus*. The results are shown in Table 2. As we can see, the reducing sugars decreased in the medium inoculated with *K. marxianus.* This result could indicate the use of sugar by *K. marxianus* for alcohol production. The amount of reducing sugars obtained with *P. ostreatus* during the SSF process is similar to the amount obtained with *T. harzianum*. According to the results after biological pretreatment, the addition of cellulase increases sharply the amount of reducing sugars in the medium.

Sample	[Reducing sugars] mg/ml
***T.harzianum* + cellulase**	448.74
T.harzianum* + cellulase + *K.marxianus	361.95
***T.reesei* + cellulase**	422.02
T.reesei* + cellulase + *K.marxianus	362.74
***A.niger* + cellulase**	451.26
A. niger* + cellulase + *K. marxianus	385.18
***P. ostreatus* + cellulase**	404.57
P. ostreatus* + cellulase + *K. marxianus	353.15

Table 2. Determination of reducing sugar present in the media after the fermentation with *K. marxianus* and in the medium without fermentation for the different pretreatments.

As observed experimentally (Table 3), the fermentation that produces higher quantity of alcohol corresponds to sample pretreated with *T. harzianum* (2.92 ± 0.11). Ethanol values obtained with *T. harzianum*, *A. niger* and *P.ostreatus* were higher than the media pretreated with *T. reesei*.

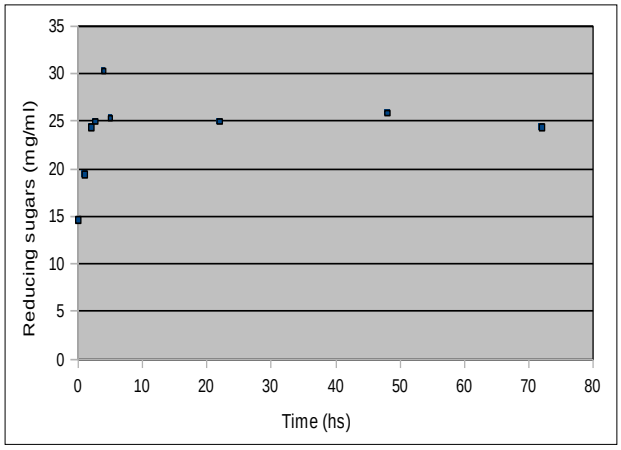
Pretreatment	Degrees of ethanol (º)
Blank	0.02
A. niger	2.27
P. ostreatus	2.01
T.reesei	0.71
T. harzianum	2.92

Table 3. Ethanol determination by gas chromatography.

3.4 Scale up of SSF

Graphic 2 shows the concentration of reducing sugars vs time. We can observe a increase in reducing sugars during the first hours and then begins to drop due to the consumption of these in fermentation. Generally, at the beginning of the process, the rate of glucose production (saccharification) is greater than the rate of glucose

consumption (fermentation). At the inflection point, both rates are equal, and when the concentration of sugars decrease, the rate of consumption is greater than the rate of production (Castaño et al, 2008). In this case, the decrease of reducing sugars at the end of the process is not very pronounced. This behavior could be due to an excess of cellulase, producing high amount of reducing sugars not allowing to observe the consumption.



Graphic 2. Reducing sugars vs time.

4 CONCLUSIONS

From the different pretreatments done, it was determined the optimal conditions for this step: pH of 5, 30ºC and the utilization of the consortium *T. harzianum -P. ostreatus (*2:1). We reported concentrations of ethanol production in the range of 2-3% (w/v), using 25U of *T. harzianum* cellulases and *K.marxianus* at 72 hours of SSF.
Previous studies report the obtention of the same percentage after 4-6 days of incubation using *T. reesei* cellulases, and *S. cerevisiae* (Hari et al., 1998, 1999).
Hari et al., 2000 reported the obtaining of 3% of ethanol (w/v) at 57.2 hours.
T. harzianum and *A. niger* will be a good combination due to the production of ethanol showed.
We can conclude that orange waste is a favorable substrate for ethanol production.

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