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Ethanol Production from Sunflower Seed Hull Hydrolysate by *Pichia stipitis* under Uncontrolled pH Conditions in a Bioreactor

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Abstract

Ethanol production from sunflower seed hull hydrolysate was evaluated using *Pichia stipitis* NRRL Y-7124 in this study. The hydrolysate was prepared with 0.7 M H_2SO_4 and a solid/liquid ratio of 1/5 (w/v) at 90 °C. Fermentation of detoxified hydrolysate was carried out in a batch bioreactor system under uncontrolled pH operation at initial pH of 6 at 30 °C. The influence of different aeration rates on the fermentability of hydrolysates was investigated. The highest ethanol accumulation, 9.66 g l^{-1}, and a yield of 0.41 g g^{-1} were achieved at the lowest tested flow rate, 2.28 $vv^{-1}min^{-1}$, from 35 g reducing sugar l^{-1}.

Key words: Ethanol, Fermentation, Hydrolysate, *Pichia stipitis,* Sunflower seed hull.

Introduction

The utilization of renewable lignocellulosic agro-industrial residues has been attracting interest due to high petrol prices and depletion of fossil fuel reserves and, more recently, increasing environmental and political pressures (Davis et al., 2005). When hydrolyzed, these lignocellulosic materials release sugars and several compounds derived from sugar and lignin degradation. The lignocellulosic hydrolysates can be used as fermentation media to obtain ethanol, xylitol, and other useful products (Mussatto, 2003). Bioconversion of lignocellulosics to ethanol requires initial dilute acid hydrolysis of hemicellulose to xylose followed by fermentation by microorganisms. The ability of *Pichia stipitis* and *Candida shehatae* yeasts to ferment xylose efficiently to ethanol has led to many investigations that use lignocellulosic hydrolysate as fermentable substrate. However, rapid and efficient fermentation of hydrolysates is limited because a range of toxic compounds in addition to monomeric sugars are generated during the hydrolysis of lignocellulosics (Palmqvist and Hahn-Hägerdal, 2000). Various crop residues rich in lignocellulosics, like wheat straw (Nigam, 2001), rice straw (Roberto et al., 1999), corn cob (Saraçoğlu-Eken and Arslan, 2000), sunflower stalks (Sharma, 2002), sunflower hulls (Sharma, 2004), and water-hyacinth (Nigam, 2002), have been exploited for ethanol production. Turkey is ideal for growing sunflowers and building oil extraction plants. The sunflower is a deciduous plant whose yearly production has increased dramatically in Turkey over the last few decades to about 950,000 t, and about 550,000 t of this production is edible oil factory extraction residue (bagasse) (Gerçel, 2002). The principal factor that must be optimized for both *P. stipitis* and *C. shehatae* is aeration rate in order to obtain maximum productivity and ethanol yield from synthetic medium or hydrolysates. In the present study, the potential use of hemicellulosic hydrolysate derived from sunflower seed hull bagasse for ethanol fermentation using *Pichia stipitis* was investigated. The choice of appropriate aeration rate for the conversion of sunflower seed hull hydrolysate into ethanol was considered.

Experiments

Materials and methods

Microorganism and growth media

Pichia stipitis NRRL Y-124 was grown at 30 °C on agar slants composed of 10 g of glucose, 3 g of yeast extract, 3 g of malt extract, 5 g of peptone, and 20 g of agar per liter. The medium described by Slininger et al. (1982) was used for growth. Inocula were prepared by transferring organisms by loop from 2-day slants to 250 ml Erlenmeyer flasks containing 100 ml of growth medium. The inoculum medium contained (per liter) 6.4 g of urea, 1.2 g of KH_2PO_4, 0.18 g of Na_2HPO_4, 10 g of yeast extracts and 50 g of D-xylose (pH 4.5). The yeast was incubated aerobically with a magnetic stirrer at 30 °C for 27 h prior to use.

Preparation of hydrolysate samples and detoxification

Sunflower seed hull bagasse taken from an oil-processing unit in Bursa province in Turkey were ground and screened to the size of 0.71-1 mm and used as feed material. The acidic hydrolysis was conducted for 3.5 h with 0.7 M H_2SO_4 and a solid/liquid ratio of 1/5 (w/v) at 90 °C. The hydrolysate was recovered after separation of solid and liquid fractions by filtration. After hydrolysis, the hydrolysate has 37 g total reducing sugar l^{-1}. This corresponds to 90% of potential hemicellulosic sugar in seed hulls. To minimize the concentrations of fermentation inhibitors, the hydrolysate samples were heated to 60 °C in a water bath for 30 min and mixed vigorously during the rapid addition of CaO until pH 10 and filtered and acidified to pH 6 with concentrated H_2SO_4 (over-liming). The sample was again filtered. Over-limed hydrolysate was supplemented with 3 g of Na_2SO_3 l^{-1} and the sample was filtered. Each time, total reducing sugar loss was compensated for with synthetic xylose addition into hydrolysate to maintain a total reducing sugar concentration of around 35-40 g l^{-1} after any detoxification application. The treated hydrolysates were further supplemented with additional nutrients to maintain the growth medium composition.

Fermentation conditions

Sunflower seed hulls were hydrolyzed with sulfuric acid and the resulting hydrolysates were used for ethanol production with the yeast *Pichia stipitis* after detoxification. All the experiments were performed in a batch culture bioreactor system, which consisted of a glass flask, a Teflon and silicone-lined top cap and a Teflon and glass impeller assembly mounted on the top cover. The flask has a working volume of 0.6 l and a bottom dimple beneath the paddle blade magnetic impeller. The entire unit can be autoclaved. The air inlet and CO_2 outlet on the top cover were equipped with air filters. Air pumped through a flowmeter was sparged into the fermentation medium from the bottom of the vessel. The bioreactor was immersed in a temperature controlled bath and the whole system was placed on a magnetic stirrer. During the experiments, temperature and agitation rate were controlled at 30 °C and 100 rev min^{-1}. Fermentation medium was inoculated with 20% (v/v) cultures. In all experiments, pH, initially adjusted to 6, was not controlled during fermentation. Four different aeration rates were tested (0, 2.88, 5.76, 7.99 $vv^{-1}min^{-1}$). Aliquots of 5 ml were drawn periodically to determine cell mass, ethanol, residual sugar and pH in the broth.

Analytical method

Biomass concentration was determined turbidometrically by a UV spectrophotometer at 600 nm. This analysis was calibrated by dry weight analysis, where 10 ml samples were taken directly from the growth media, centrifuged, washed with distilled water and dried at 90 °C for approximately 24 h. Total reducing sugars were quantified using dinitrosalicylic acid (Miller, 1959). Ethanol was measured by the dichromate oxidation method (Hormitz, 1980). The ethanol yields ($Y_{P/S}$) were based on consumed total reducing sugar concentration at maximum ethanol concentration. The volumetric productivity (Q_P) was calculated as the maximum ethanol produced divided by the time to achieve maximum ethanol.

Results and Discussion

In this study, sunflower seed hull hemicellulose was degraded to sugars using sulfuric acid. Recovery of sugars from hemicellulose of approximately 90% was achieved by using a relatively low temperature (90 °C) and mild acid concentration (0.7 M). This temperature was chosen since increased hydrolysis temperature has been reported to increase inhibition products (Larsson et al., 1999). Detoxified sunflower seed hull hydrolysates having initial reducing sugar

concentrations around 35-40 g l^{-1} were used as substrates for the production of ethanol. Figures 1 and 2 show the time course for the growth and ethanol concentration in hydrolysates at initial pH 6 at various aeration rates. The fermentation parameters are summarized in Table 1. It seems that both biomass growth and ethanol formation are sensitive to the amount of oxygen supplied. When air flow rate is zero, insufficient aeration leads to very small biomass and ethanol formation. During all the other experimental runs, an oxygen supply stimulated significant growth and ethanol formation depending on aeration rate. Increasing the tested air flow rate from 2.28 to 7.99 $vv^{-1}min^{-1}$ slightly improved cell growth in hydrolysates. In contrast, the highest ethanol accumulation, 9.66 g l^{-1}, and productivity rate (Q_P), 0.18 g $l^{-1}h^{-1}$, and a yield of 0.41 g g^{-1} for sunflower seed hull hydrolysate were achieved at the lowest tested flow rate, 2.28 $vv^{-1}min^{-1}$. Further increases in flow rate produced less ethanol. This suggests that the degree of aeration has to be at a certain threshold level before ethanol production is diminished. As seen from Table 1, there is a negative correlation between ethanol yield and oxygen supply, which indicates that excessive aeration reduces the ethanol yield because of either product oxidation or cell growth. This observation is in agreement with some previous results (Grootjen et al., 1990; Nigam, 2002). Figures 3-6 show the substrate utilization, ethanol formation and changes in the pH of sunflower seed hull hydrolysates. In spite of the complex nature of hydrolysate, no strong diauxic pattern of total reducing sugar utilization was observed. It is evident that sugar consumption, pH change and oxygen supply rate are closely related. While substrate consumption was only 9% for fermentation without aeration, it attains a level of 75%-78% for other runs with various flow rates. It is reported that cell growth in lignocellulosic hydrolysates is dependent on pH due to the degree of toxicity (Palmqvist and Hahn-Hägerdal, 2000). That is why most hemicellulosic hydrolysates fermentations have been conducted at pH values above the optimal values of 4 to 5.5 in order to minimize inhibitory effects. A value of 6 was chosen as the initial pH of fermentations for that reason in the present study. In hydrolysate media in our work under uncontrolled pH operation, pH has first a tendency to decrease from 6 to between 5.3 and 5.38 with cultivation time in all runs (Figure 7). In the experiment without aeration, pH showed no change during the fermentation after that initial drop. However, with increasing air supply, an increase in pH took place until the end of the fermentation in all the other runs. The observation of different pH profiles with aeration rate may confirm a change in metabolism, which may result from different distribution and transportation of nutrients to the cells (Feng et al., 2003). Figures 3-7 clearly indicate that a slow increase in pH at the second stage of fermentation favors ethanol formation. It can be suggested that the aeration rate capable of maintaining pH for longer around the initial optimum value helps to overcome the inhibitory effects and produce more alcohol under uncontrolled pH operation. The fermentation of many treated hydrolysates has been examined by different investigators using various sugar utilizing yeasts and the results are shown in Table 2. From a comparison it is clear that our results are within the range of values reported by others. On the basis of our data, we may conclude that the hydrolysate derived from sunflower seed hulls has a significant potential for ethanol production by using *P. stipitis*. Furthermore, it has been shown in this study that the choice of appropriate aeration may lead to considerable ethanol formation even under non-controlled pH operation.

Table 1. Effect of aeration rates on ethanol fermentation of sunflower seed hull hydrolysates by *Pichia stipitis*.

	Aeration rate (v v^{-1} min^{-1})			
	0	2.88	5.76	7.99
Ethanol (g l^{-1})	1.61	9.66	6.90	4.14
Q_P (g $l^{-1}h^{-1}$)	0.05	0.18	0.12	0.11
$Y_{P/S}$ (g g^{-1})	0.46	0.41	0.26	0.17
% utilized sugar	9	78	76	75

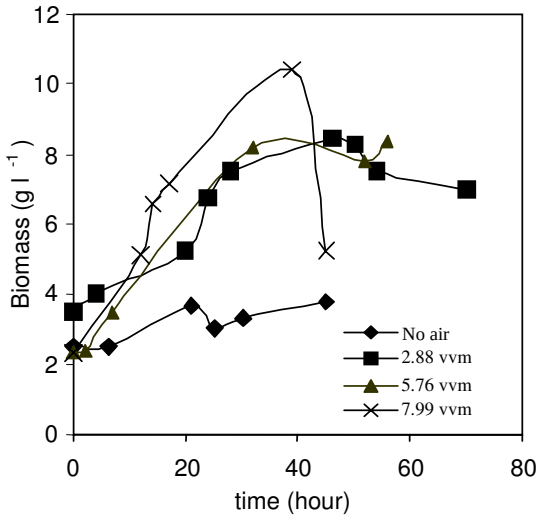

Figure 1. The effect of different aeration rates on biomass growth during fermentation of sunflower seed hull hydrolysate by *Pichia stipitis*.

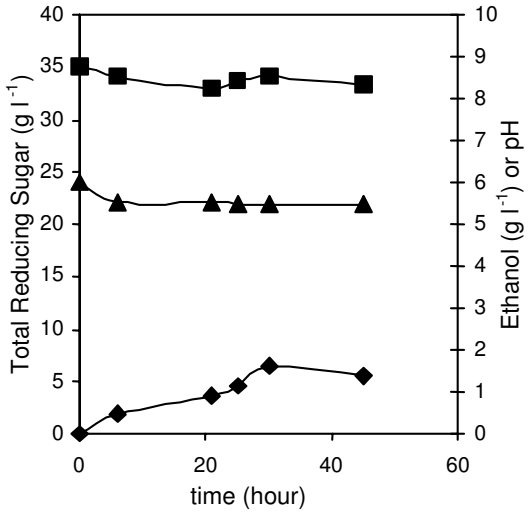

Figure 3. Ethanol production from sunflower seed hull hydrolysate by *Pichia stipitis* at 0 v v^{-1} m^{-1} aeration rate conditions. (■) Reducing sugar concentration, (▲) pH, (♦) Ethanol concentration.

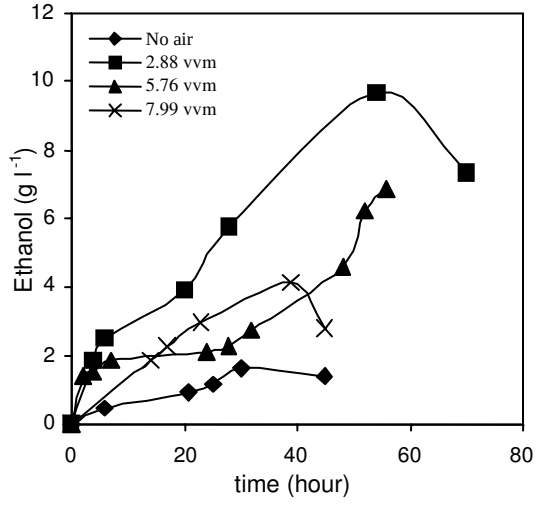

Figure 2. The effect of different aeration rates on ethanol formation during fermentation of sunflower seed hull hydrolysate by *Pichia stipitis*.

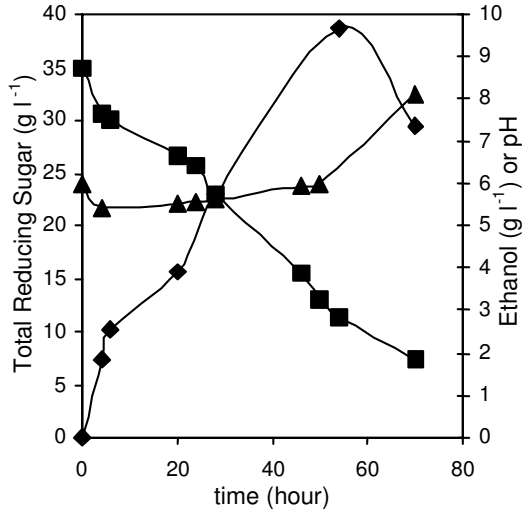

Figure 4. Ethanol production from sunflower seed hull hydrolysate by *Pichia stipitis* at 2.88 v v^{-1} m^{-1} aeration rate conditions. (■) Reducing sugar concentration, (▲) pH, (♦) Ethanol concentration.

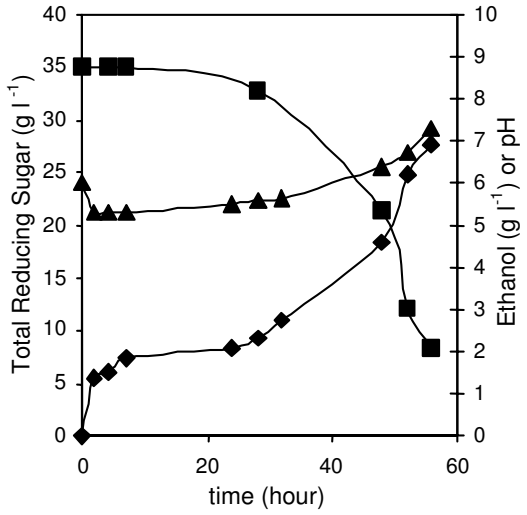

Figure 5. Ethanol production from sunflower seed hull hydrolysate by *Pichia stipitis* at 5.76 v v^{-1} m^{-1} aeration rate conditions. (■) Reducing sugar concentration, (▲) pH, (♦) Ethanol concentration.

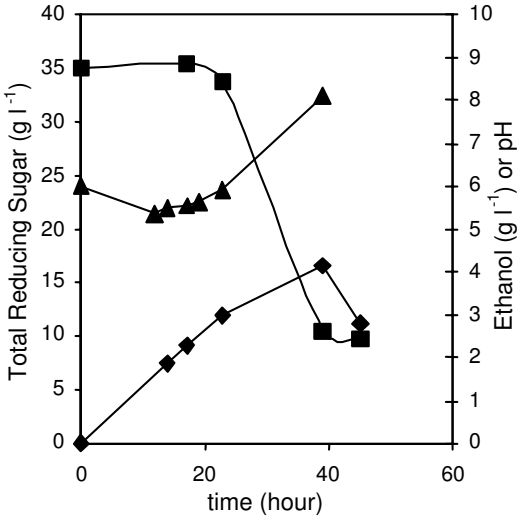

Figure 6. Ethanol production from sunflower seed hull hydrolysate by *Pichia stipitis* at 7.99 v v^{-1} m^{-1} aeration rate conditions. (■) Reducing sugar concentration, (▲) pH, (♦) Ethanol concentration.

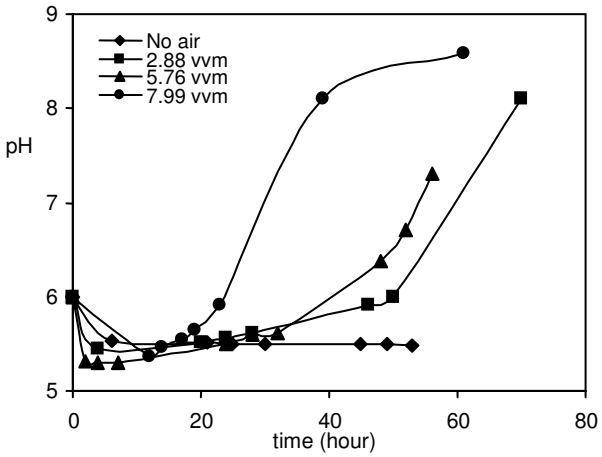

Figure 7. The effect of aeration rates on pH value during fermentation of sunflower seed hull hydrolysate by *Pichia stipitis* at initial pH of 6.

Table 2. Comparison of literature in ethanol fermentation using sunflower seed hull hydrolysate by *Pichia stipitis.*

Hydrolysate	Yeast	Substrate	Ethanol	Q_P	$Y_{P/S}$	References
		(g l^{-1})	(g l^{-1})	(g $l^{-1}h^{-1}$)	(g g^{-1})	
Wheat straw	*P. stipitis*	60.0	12.90	0.30	0.36	Nigam 2001
Sugar Cane Bagasse	*P. stipitis*	48.5	15.00	0.25	0.38	van Zyl 1988
Water-Hyacinth	*P. stipitis*	67.5	18.00	0.18	0.35	Nigam 2002
Sunflower stalks	*S. cerevisiae*	40.0	15.12	0.84	0.44	Sharma 2002
Sunflower hulls	*S. cerevisiae*	40.0	15.81	0.88	0.45	Sharma 2004
Sunflower seed hull	*P. stipitis*	35.0	9.66	0.18	0.41	This study

Nomenclature

$vv^{-1}min^{-1}$ aeration rate (ml of air / ml of solution / min)

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