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Inhibitory Effect of High Concentrations of Furfural on Industrial Strain of *Saccharomyces cerevisiae*

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ABSTRACT: Bioethanol made from lignocelluloses biomass resources, provides unique environmental, economic and strategic benefits, when compared to gasoline as automobile fuel. To be able to substitute gasoline by bioethanol, one should over come many obstacles, including the production of furfural and hydroxyl-methyl furfural, which are produced when cellulosic materials are treated in the presence of acids in high temperature and pressure to produce simple sugars. These inhibitory compounds have a profound negative effect on the growth of ethanol producing yeasts and their ethanol production. *Saccharomyces cerevisiae* Lalvin EC1118™, an industrial ethanologenic strain, was used in media with high concentrations of furfural (i.e. 4, 5 and 6 g/L) to study the tolerance it shows against this compound. Results showed that both the amount of growth and ethanol production were decreased when furfural was present in the media. The amount of decrease was higher in the concentration of 6 g/L of furfural than 4 and 5 g/L.

Key words: Bioethanol, Growth Yield, Ethanol Yield, Furfural, *Saccharomyces cerevisiae*

INTRODUCTION

The environmental pollution is one of the major problems in the world. The usage of fossil fuels, directly and indirectly; pollute the air, soil and water. Production of polluting gases such as CO, NOXs, cyclic compounds, SO_2 and others, are the known direct risks of using these fossil fuels. As the indirect risks of oil based fuels, Methyl-*tert*-butyl ether (MTBE) may be mentioned. MTBE, which is one of the gasoline additives for enhancing the combustion efficiency of engines (Shahidi Bonjar, 2007), is the most commonly used oxygenating compound. MTBE has low cost, high-octane level, and ease of blending with gasoline, but is carcinogenic and can contaminate the soil, water, and ground water (An Kampbell and Sewell, 2002). The increasing concerns about environmental protection, has led to the use of bioethanol as sole fuel, or a blend with gasoline. Ethanol is one of the most important renewable fuels contributing to the reduction of negative environmental impacts generated by the worldwide utilization of fossil fuels. Production of ethanol from agricultural and biodegradable wastes, such as lignocelluloses materials, provides a viable solution to multiple environmental problems simultaneously creating link for waste treatment and renewable energy production as well (Patle and Banwari, 2007). The combustion of ethanol originating from lignocelluloses materials provides for no net atmospheric release of carbon dioxide, due to the respiration of CO_2 during forest growth and maturation. Ethanol has been reported that, in ground water and soil mixtures, can be rapidly degraded both aerobically (100 ml/L in 7 days) and anaerobically (100 mg/L in 3-25 days), thus not harmful to the biotope as long as it is not present in concentrations directly toxic to microorganisms.

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The half-time of ethanol in surface water is 6.5 to 26 hours. While ethanol releases volatile organic compounds (VOC) due to its low vapor pressure, degradation of ethanol in the atmosphere is also predicted to be rapid (Cardona and Sánchez, 2007). Bioconversion of lignocelluloses biomass to ethanol requires hydrolysis of the two carbohydrate polymers to their constituent monomeric sugars prior to microbial fermentation. Lignocelluloses hydrolysis has been achieved using either acid (Lee *et al.*, 2000) or enzymes (Sreenath and Jeffries, 2000). Acid hydrolysis is an easy and cheap method to derive sugars from lignocelluloses materials. An important problem in fermentative conversion of lignocelluloses to the ethanol is the severe inhibitory effects often exerted by some byproducts of lignocelluloses hydrolysates such as furfural (Olsson and Hähen-Hägerdal, 1996 and Larsson *et al.*, 2000). Furfural has been reported to have inhibitory effects on the specific growth rate, as well as fermentation rate of yeasts (Palmqvist *et al.,* 1999a). In order to avoid such inhibition, various treatments for the detoxification of fermentation inhibitors have been investigated (Klinke *et al.,* 2004). *Saccharomyces cerevisiae* is a common microorganism used for ethanol fermentation industry because of its good tolerance against inhibitors, such as furfural than other candidate microorganisms (Olsson and Hähen-Hägerdal, 1996).

In the present paper, we attempt to show inhibitory effects of different concentrations of furfural on an industrial strain of *Saccharomyces cerevisiae* using batch fermentation with synthetic medium under aerobic and anaerobic conditions.

MATERIALS & METHODS

The industrial strain of *Saccharomyces cerevisiae* Lalvin EC1118™ from Lallomands (Scott Laboratories, Canada) was used as the test microorganism. The yeast was stored at 4°C on Potato Dextrose Agar (PDA) containing (w/v): Potato (Shaved, boiled and filtered) 30%, Glucose 3% and Agar 1.5%. Pre-culture medium was a synthetic medium containing (w/v): Dextrose 3%, Yeast extract 1%, Ammonium Phosphate 0.06%, Ammonium Sulfate 0.12%. The pH of the media was adjusted to 5.3 by 1 M HCl. Glucose was autoclaved separately. Production medium was a synthetic medium containing (w/v): Glucose 20%, Yeast extract 1%, Ammonium Phosphate 0.06%, Ammonium Sulfate 0.12%, pH 5.3. Before inoculation, different concentrations of newly distilled pure furfural, between 0-6 (g/L), was added. The yeast was maintained on PDA slants. After 48 h incubation time at 30°C, one loop-full of cells was transferred to 250-ml Erlenmeyer flasks, containing 50 ml of the pre-culture medium, and incubated on rotary shaker incubator (Clim-O-Shake, Switzerland) at 30°C, 150 rev. min^{-1} for 20 h. The pre-culture was transferred at a proportion of 10% (v/v) to the production medium. Furfural was added to the production medium at concentrations of 4.0, 5.0 and 6.0 g/L. Since the growth of the yeast cells should take place in aerobic condition, for the first 8 hours, the flasks were placed on the shaker-incubator, at 30°C, 150 rev. min^{-1}. After this period, the cultures were aseptically transferred to 100 ml Universal bottles, equipped with rubber stoppers, to which, sterile syringe needles were inserted to release the pressure of produced CO_2. The bottles were incubated at 30°C for further 40 h to complete the fermentation under anaerobic condition (Palmqvist *et al.,* 1999a).

The amount of biomass was measured by determining the dry weight of cells obtained by centrifugation of cultures at 10'000 rev. min^{-1} for 10 min., and drying it at 95°C for 24 h. Where applicable, the amount of growth was also measured by cell count done by Neobar type counting chamber and/or by measurements of turbidity of cultures against non-inoculated blank samples at 620 nm. by Unicam 8620 UV/VIS spectrometer. Ethanol quantification was achieved by gas chromatography on 14A Shimadzu with an on-column injector system, and flame ionization detector (at 230 °C). Separation was effected in a 30 m Carbowax 20M column (diameter 0.2 mm). The initial temperature was programmed to be 50 °C, which was raised to 200 °C as final temperature with a rate of 5 °C/min. The carrier gas was N_2 (50 ml/min). Glucose concentration was measured by enzymatic method (Kit D-Glucose, Chimenzyme, Iran).

Ethanol yield ($Y_{P/S}$) was calculated by the formula (1):

$$Y_{P/S} = \frac{[EtOH](g/L)}{[Glucose](g/L)} \quad (1)$$

Where, [EtOH] was the concentration of ethanol, produced after 48 h and [Glucose] was the initial concentration of the glucose in the production medium. Biomass yield ($Y_{X/S}$) was calculated by formula (2) as below:

$$Y_{X/S} = \frac{[Biomass](g/L)}{[Glucose](g/L)} \quad (2)$$

Where, [Biomass] was the dry weight of yeast cells, obtained after 48 h., and [Glucose] was the initial concentration of the glucose in the production medium (Oliva, *et al.*, 2006).Special growth rate (μ) of the microorganism was calculated by formula (3):

$$\mu_{OD} = \frac{(OD_2 - OD_1)}{(t_2 - t_1)} \times \frac{1}{OD_1} \quad (3)$$

Where, OD_1 and OD_2 are the turbidity of yeast cultures at t_1 (time 1) and t_2 (time 2) and μ_{OD} is the special growth rate of the yeast, as measured by its differences in culture turbidity.

RESULTS & DISCUSSION

The effect of different concentrations of furfural on *Saccharomyces cerevisiae* EC1118™ was studied. The concentrations chosen were within the range of 0-6 g/L. The glucose consumption, biomass yields ($Y_{X/S}$), ethanol yields ($Y_{P/S}$) and special growth rate of the yeast (μ), were determined in the fermentation course of ethanol. The performance of the yeast, regarding ethanol and biomass production, in these concentrations during the batch cultivation is displayed in Fig. 1. The highest glucose consumption rate after 48 h was obtained for furfural free medium which showed to be 93% of the initial glucose. When to the media was added 4 ,5 and 6 g/L furfural, this figure was decreased to about 60%, 51% and 33%, respectively. This indicated the presence of a negative effect, exerted by high concentrations of furfural, on the glucose uptake and metabolic system.

Depicted results in Fig.1 showed that, while the mean value of produced biomass in furfural free medium was about 0.53 g/100mL, in media containing 4, 5 and 6 g/L furfural, substantial reduction of produced biomass to about 0.31, 0.22 and 0.17 g/100 ml of the medium, is taken place,

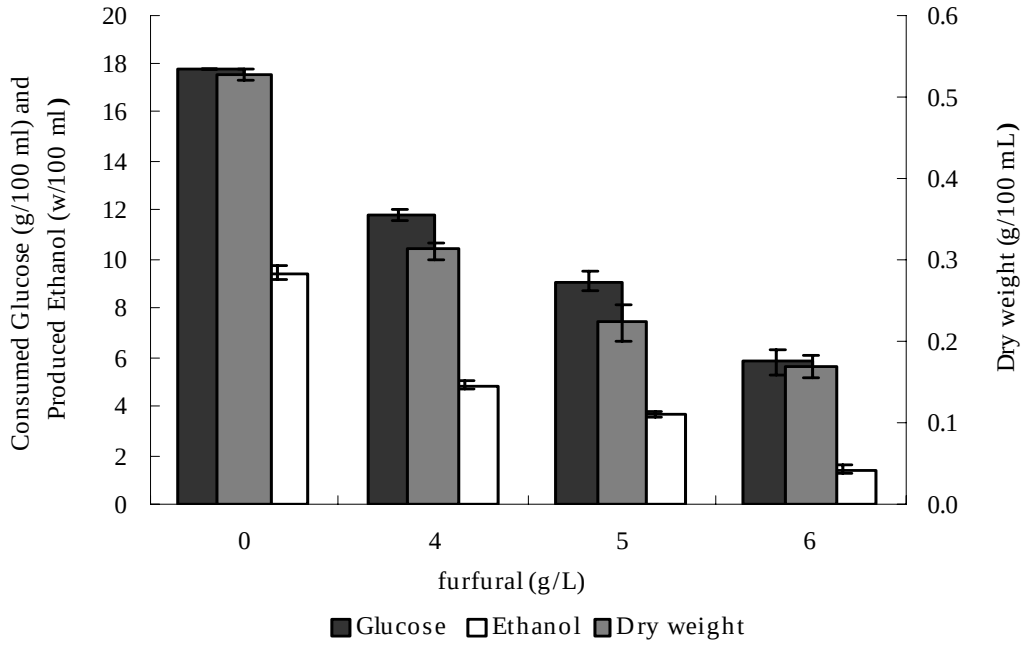

Fig. 1. Effect of different concentrations of furfural on: glucose consumption; ethanol and biomass dry weight production by *S. cerevisiae* after 48 h. Error bars represent standard deviation of three replicates

respectively. The maximum ethanol concentration produced in furfural free medium was 9.4% (w/v), while in the media containing 4, 5 and 6 g/L furfural, ethanol production was reduced to 4.8%, 3.7% and 1.4%, respectively. This indicated the sensitivity of yeast cells, to the increased furfural-related toxicity of the medium.

By comparing the yields of growth and ethanol production in the above mentioned conditions, furfural in high concentrations showed to be a strong inhibitor for the conversion of glucose to biomass and ethanol by *S. cerevisiae* strain EC1118 (Fig. 2), since according to the formula (3), the "yield", indicates the amount of produced product (ethanol and biomass) to the consumed substrate (glucose).

A normal behavior of ethanol producing cultures of *S. cerevisiae* is that the cell number increases exponentially in the first part of the growth, where aerobic condition presents, and cells are produced as the metabolic output of the glucose consumption, while in the second part of the culture, where anaerobic condition is established intentionally, the cells cease to divide and even decrease in number, and shift to production of ethanol, as metabolic output of consumption of glucose. This phenomenon, regarded as "Pasteur effect", is obvious in the Fig. 3, where the cultures were void of furfural. The turbidity of culture increased in the first 8 h very rapidly, in the first part, but stopped and declined as time passed by. By adding the furfural to the media, the initial growth of the cells, decreased substantially, as is presented in the Table 1. The interesting point was that in the anaerobic part of the fermentation, in the presence of furfural, the growth was not stopped and the cell mass increased slowly. However, the cell concentration did not reach the amount, where the furfural free medium reached. There are some researches, in which the conversion of furfural to furfuryl alcohol in the anaerobic condition is reported (Palmqvist *et al.*,1999b).

Table 1. Special growth coefficient of *S. cerevisiae* in theabsence and presence of furfural

	Concentration of furfural g/L			
	0	4	5	6
$\mu_{(0\text{-}8)}$ (aerobic)	2.05	0.07	0.106	0.097
$\mu_{(8\text{-}48)}$ (anaerobic)	-0.005	0.076	0.048	0.024

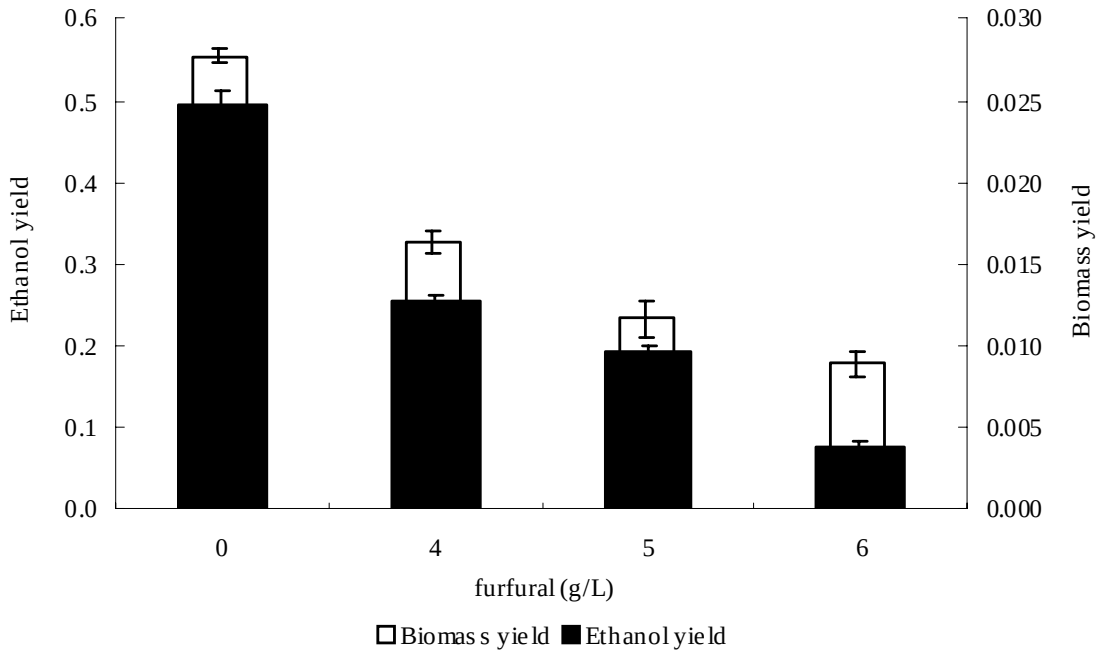

Fig. 2. Comparison between the yields of ethanol and biomass production in the different furfural concentrations after 48 h by *S. cerevisiae*. Error bars represent standard deviation of three replicates

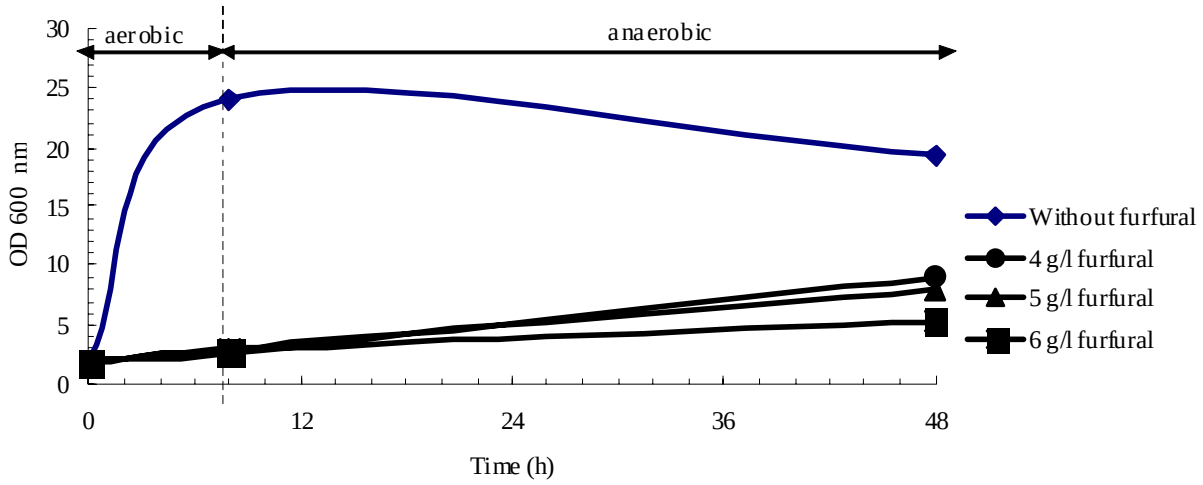

Fig. 3. Effect of furfural concentration on growth of yeast, as measured by cultures' turbidity, in the aerobic and anaerobic parts of the ethanol fermentation

The furfural inhibitory effect on growth in anaerobic condition was enhanced by raising the furfural concentration. These results agree with those obtained by Palmqvist et al. (1999b) with *S. cerevisiae* in the presence of 10 g/L of acetic acid and 3 g/L of furfural, suggesting that the presence of furfural, and its reduction to furfuryl alcohol, prevents the formation of glycerol which is necessary to regenerate the excess NADH during fermentation, and to maintain the intracellular redox balance. This decrease in the formation of by-products could explain the positive effect on biomass production in the presence of furfural (Palmqvist *et al.*, 1999b).

The presence of toxic products in fermentation media can produce the following effects: (i) reduction in the specific growth rate (Navarro, 1994 and Olsson, 1996) (ii) decrease in the volumetric productivity of ethanol (Navarro, 1994 and Larsson, 2000; (iii) decrease in the specific productivity of ethanol (Taherzadeh, 2000); (iv) decrease in biomass production (Olsson, 1996). The tolerance to aldehyde compounds is most likely due to the ability of microorganisms to convert these compounds to the corresponding less inhibitory alcohols. In anaerobic environments, most ethanologenic microorganisms (e.g. *S. cerevisiae*) reduce furans to their corresponding alcoholmoieties as a means of detoxification (Taherzadeh, 1999 and Villa, 1992). However, furans at high concentrations exert an inhibitory effect, interfering with glycolytic enzymes and macromolecule synthesis (Taherzadeh, 1999 and Patle, 2007).

CONCLUSION

The objectives of this study were to investigate the inhibitory effects of high concentrations of furfural (i.e. 4, 5 and 6 g/L) on the fermentative performance of *S. cerevisiae* strain EC1118 in batch cultures and to determine the amount of glucose consumption, biomass and ethanol production yield of this strain. The results showed that in the applied concentrations of furfural, *S. cerevisiae* strain EC1118 losses its metabolic activity (as could be shown by glucose consumption) and ethanol production capacity. For an economic conversion of lignocelluloses materials to ethanol, further investigations for finding a suitably ethanologenic strain, showing tolerance against furfural, is of great importance.

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