Influence of Furfural on the Recombinant *Zymomonas mobilis* Strain CP4 (pZB5) for Ethanol Production

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Abstract: The toxicity of furfural as an inhibitor of growth and ethanol production by recombinant *Zymomonas mobilis* strain CP4 (pZB5) was investigated by adding furfural 0.475 g/l and 1.9 g/l in the fermentation media. The inhibitory effects of furfural were observed with a decrease of 30% and 60% (w/v) in biomass production and 19% and 76% (v/v) in ethanol production, respectively. [The Journal of American Science. 2005;1(1):24-27].

Key Words: ethanol; furfural; glucose; inhibitor; *Zymomonas mobilis*; xylose

1. Introduction

Traditionally, ethanol is been produced in batch fermentations with yeast strains that cannot tolerate high concentrations of ethanol. Strain improvement is necessary to obtain alcohol-tolerant strains for fermentation. *Z. mobilis*, a gram-negative bacterium, is considered an alternative organism for large-scale fuel ethanol production (Mohagheghi, 2004).

The complex of lignocellulosic components in plant fiber presents difficulties for conversion of wood to ethanol. It is important to study the effects on fermentation parameters of all the components that result from hydrolysis and separation and know how to deal with toxic compounds.

Three major pretreatment alternatives exist: milling, steam explosion, and dilute acid hydrolysis. The latter option maximizes use of the hemicellulose fraction of the wood (Boussaid, 2000). Typically, wood residues are treated at temperatures ranging from 200°C to 220°C, with cooking times of 3 to 5 minutes and 2% to 3% of SO_2 (v/v). Another condition is 170°C, 15-60 minutes cooking and 0.01-0.1% (v/v) of H_2SO_4 (Um *et al*., 2001).

The components of hemicellulose are converted to pentoses and degradation products, according to the following reactions (Figure 1):

Pentosan -----(H₂O)----➔   pentose (xylose)---(- 3 H₂O)--➔   furfural
Figure 1. Conversion reactions
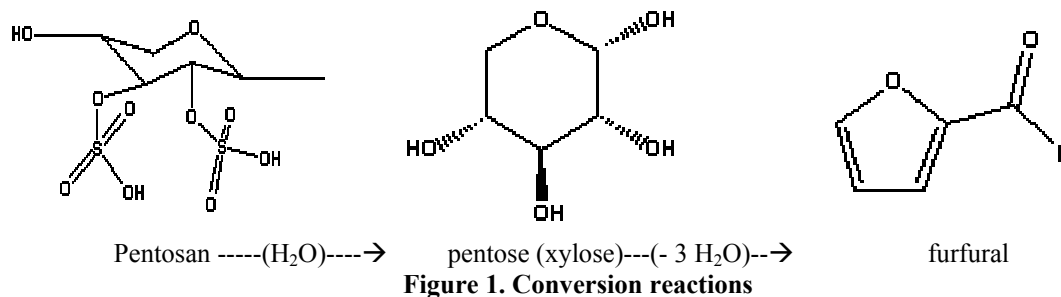
Hydroxymethylfurfural (HMF) is also produced from hexose sugars under high temperature acidic conditions.

Morphological changes are observed by light microscopy during growth of *Z. mobilis* at partially inhibitory concentrations of aldehydes. Cells either appear as elongated or forming short chains after 6 hours compared with normal cells. After 24 h, cells are swollen and rounded (Zaldivar, 1999).

Shahab and Hadi (1995) demonstrate that furfural reacted primarily with AT base-pair sequences in double-stranded DNA causing strand breaks via spontaneous depurination and depyrimidination.

2. Material and Methods

Recombinant *Z. mobilis* strain CP4 (pZB5), which contains the *Escherichia coli* genes for xylose assimilation and pentose metabolism (transketolase, transdolase) on the plasmid pZB5 together with the gene for tetracycline resistance (Zhang, 1995), was used in these experiments.

Growth media for the recombinant *Z. mobilis* strain was as follows: glucose and xylose concentrations as specified, $(NH_4)_2SO_4$ (1 g/l in media containing 10 g/l glucose and 10 g/l xylose); KH_2PO_4 (2 g/l); $MgSO_4.7H_2O$ (1 g/l); yeast extract (10 g/l). Media were sterilized by autoclaving at 121°C for 15 minutes. Tetracycline was added aseptically to the sterile media at room temperature at a concentration of 10 mg/l as selection pressure for plasmid (pZB5) maintenance.

All inocula were prepared in stationary shake flasks incubated at 30°C at an inoculation density of 0.004 g/l.

Experiments were conducted in 500 ml shake flasks with a working volume of 200 ml. Control fermentations without furfural were carried out simultaneously, under exactly the same conditions, using the same inoculum preparation to compare with those fermentations containing furfural. Environmental conditions were controlled at a temperature of 30°C and an agitation rate of 200 rpm.

Furfural at (0.475 g/l) and at (1.9 g/l) were added 10 hours after inoculation, between lag and exponential phases. Samples were withdrawn at regular intervals for OD measurements and were filtered (Whatman Syringe filters, Fisher Scientific, pore size 0.2 μm, Nylon) for xylose, glucose, ethanol and furfural analysis. The filtered solutions were stored at –20°C prior to analysis. Each of the fermentations was run in duplicate.

Biomass concentrations were determined by OD measurements (at 600 nm) using a spectrophotometer (Perkin Elmer Lambda 3B UV/VIS) and converted to DCW by use of a calibration curve.

Sugars and ethanol concentrations were determined in the filtered supernatants using a Waters HPLC with an Aminex HPX-87H column (BioRad, Hercules, CA) with 5 mM H_2SO_4 (at 65° C, 0.6 ml/min) as the mobile phase.

UV spectra were used as a rapid and convenient method to estimate total furans (HMF and furfural). Many of the lignin and sugar degradation products absorb in the UV region, all of which contain a single prominent peak at approximately 278 nm, shoulders at 320 and 220 nm, and end absorbance continuing off-scale at around 220 nm. The following equation was used to estimate furfural content: $A_{284}-A_{320}=0.127*$Total furfural (mg/l) + 0.05 (Martinez *et al.*, 2000).

3. Results (Figures 2-6)

The volumetric sugar consumption rates, Qs, are shown in Table 1. Ethanol is produced after 15 hours. However, the final concentration is reduced by 19.04% and 76.19% compared to control by using 0.475 g/l and 1.9 g/l furfural, respectively. Overall volumetric ethanol productivity, Qetoh, values are calculated using samples acquired at 38 hours.

The required nutrients for the microorganism in the culture media are modified by furfural, so that the production of biomass (Y_{xs}) is affected as shown in Table 1. Inhibition of the fermentation by furfural has been reported to decrease with increasing cell mass concentration (Boyer et al., 1992; Navarro, 1994). With the increase of furfural, the consumption of sugars Q_s and the relation with ethanol production Y_{ps} are reduced due to the morphological changes in the cell.

Furfural concentrations of 0.475 g/l and 1.9 g/l cause considerable, but not complete inhibition of biomass production. This toxic compound affects growth in the exponential phase. The specific growth rate (μ) decrease by 29% (w/v) in the experiment containing 0.475 g furfural /l and 73% (w/v) at the higher treatment level.

Glucose is completely consumed in control and 0.475 g/l furfural fermentations. However, due to the toxicity of the furfural in the mixture of 1.9 g/l furfural and 5 g/l glucose, xylose as well as glucose is utilized at lower rate thus resulting in a lower production of ethanol (Figure 5) .
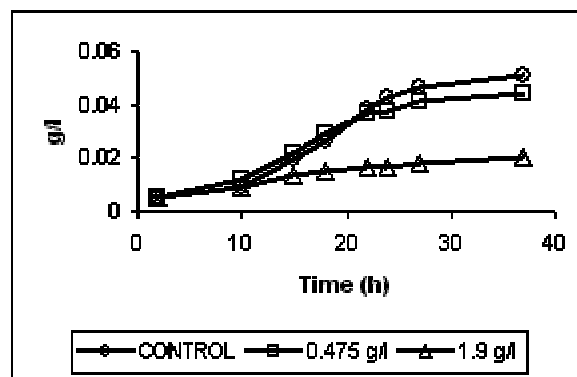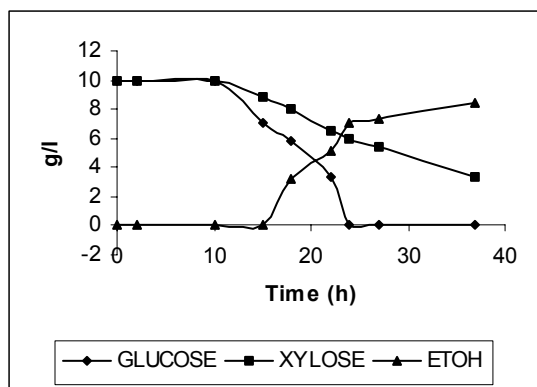

Figure 2. Biomass production.

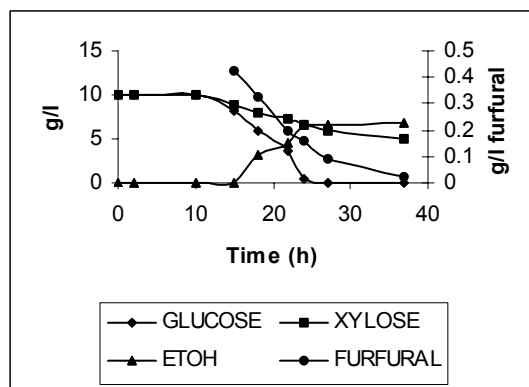**Figure 3. Consumption of sugars and ethanol production in control fermentation.**

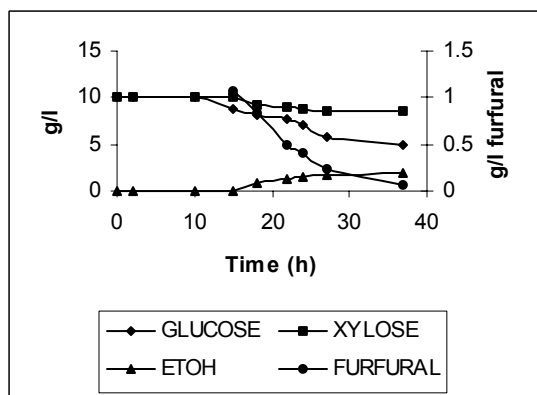**Figure 4. Consumption of sugars and ethanol production using 0.475 g/l of furfural.**

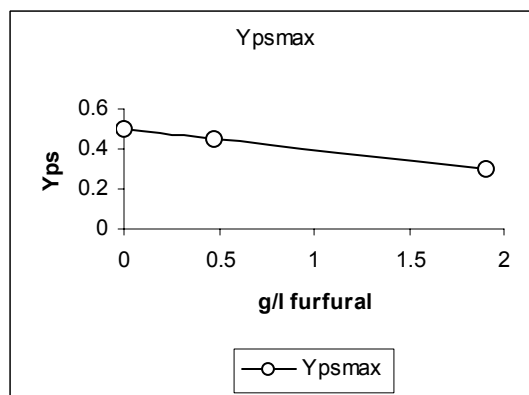**Figure 5. Consumption of sugars and production of ethanol using 1.9 g/l of furfural.**

Figure 6. Ypsmax=f (furfural).

Table 1. Fermentation parameters

Fermentation furfural g/l	Qs substrate g/time	Qetoh product g/time	Yxs cell mass/substrate g/g	Yps product/substrate g/g	μ hr^{-1}
Control	0.45	0.22	0.00271	0.5	0.089
0.475	0.40	0.18	0.00261	0.45	0.063
1.9	0.17	0.05	0.00226	0.3	0.024

Table 2. Inhibition of ethanol production by furfural (Zaldivar and Ingram, 2000)

CONCENTRATION (g/l)	INHIBITION (%)	MICROORGANISM	REFERENCE
1.3	9	*Pichia stipitis*	Tran et al, 1986
4.51	96	*E. coli* pL01297b	Beall et al., 1991
0.95	40	*Zymomonas mobilis*	Ranatunga et al., 1997
1.9	76	*Zymomonas mobilis*	This work
2.8	-1	*S. cerevisiae*	Palmqvist et al., 1999
4.0	79	*S.cerevisiae* (anaerobic)	Palmqvist et al., 1999
3.5	-38	*E. coli* LY01	Zaldivar J et al., 1999

4. Discussions

Ranatunga *et al* (1997) conducted a similar study in which inhibition of ethanol production was reported to be 4% using the same strain of *Z. mobilis* as in this study. The substrate in their work consisted of the pretreated liquid hydrolyzate prepared from the mixture of red oak, white oak, and yellow-poplar. The hydrolyzated mixture contained the following components: glucose (2.40 ± 0.48 mg/ml), xylose (44.75 ± 2.02 mg/ml), acetic acid (9.22 ± 0.43 g/l), and furfural (0.91 ± 0.051 g/l). Such substrate composition was different from the one used in present work [50% (w/v) of glucose, 50% (w/v) of xylose, no acetic acid and 1.9 g/l of furfural].

Previous work indicated that 1.3 g/l furfural might be found in hydrolysate liquor from yellow poplar sawdust (Hayward *et al.*, 1999). In our research Qetoh decreased compared with the reference fermentation when the furfural concentration increased above 1.9 g/l, indicating that when the concentration of furfural was increased beyond 1.9 g/l the energy reserves of the cell were depleted, because the toxicity of the furfural did not allow *Z. mobilis* to metabolize sugars to ethanol. (Campbell, 1996).

Data from this study and others have been incorporated into Table 2, that presents a comparison of furfural inhibition studies using several fermentative microorganisms.

Despite differences in experimental protocols and metabolic pathways, *Z. mobilis* is one the least tolerant microorganisms. These studies suggest that the complete removal of furfural in the hydrolysate liquor may be necessary for a biomass-to-fuel process.

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