Ethanol Inhibition Kinetics of *Kluyveromyces marxianus* Grown on Jerusalem Artichoke Juice

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The kinetics of ethanol inhibition on cell growth and ethanol production by *Kluyveromyces marxianus* UCD (FST) 55-82 were studied during batch growth. The liquid medium contained 10% (wt/vol) inulin-type sugars derived from an extract of Jerusalem artichoke (*Helianthus tuberosus*) tubers, supplemented with small amounts of Tween 80, oleic acid, and corn steep liquor. Initial ethanol concentrations ranging from 0 to 80 g/liter in the liquid medium were used to study the inhibitory effect of ethanol on the following parameters: maximum specific growth rate ($\mu_{max}$), cell and ethanol yields, and sugar utilization. It was found that as the initial ethanol concentration increased from 0 to 80 g/liter, and maximum specific growth rate of *K. marxianus* cells decreased from 0.42 to 0.09 h\(^{-1}\), whereas the ethanol and cell yields and sugar utilization remained almost constant. A simple kinetic model was used to correlate the $\mu_{max}$ results and the rates of cell and ethanol production, and the appropriate constants were evaluated.

Current research in the development of alternative fuels has led to the investigation of a wide variety of carbohydrate materials from which ethanol may be derived (8, 12, 15). The Jerusalem artichoke (*Helianthus tuberosus*) is attracting attention as a possible carbohydrate resource for the simultaneous production of ethanol and single-cell protein due to its several distinct advantages over the traditional agricultural crops (10, 16–20; Margaritis and Bajpai, Appl. Environ. Microbiol., in press). These advantages include minimal or no fertilizer requirements, good growth in poor soil, high tolerance to frost and plant diseases, and very high carbohydrate yields per acre (5–7).

Margaritis et al. (16) have reported optimization studies for the bioconversion of Jerusalem artichoke tubers to ethanol and single-cell protein, and Margaritis and Bajpai (18) reported studies on batch fermentation kinetics of two strains of *Kluyveromyces marxianus* and one strain of *Saccharomyces rosei* grown on Jerusalem artichoke tuber extract. These studies involved the fermentation of Jerusalem artichoke extract containing about 10% total sugars, which produced a maximum ethanol concentration of about 4.8%. The production of ethanol in high concentrations in the fermentor would be advantageous, since distillation would be reduced and contamination risks during fermentation minimized. An efficient continuous process would require a yeast strain able to both grow and ferment at high ethanol concentrations. Previous work in our laboratory (16, 18) has shown that the yeast *K. marxianus* UCD (FST) 55-82 grows very well in the inulin-type sugars present in the extract derived from the tubers of Jerusalem artichoke. Inulin is a polymer of about 2 to 35 fructose units with 1 glucose unit at the end of the molecule (7).

In this paper we present the results on ethanol inhibition kinetics of *K. marxianus* UCD (FST) 55-82 grown on the extract derived from Jerusalem artichoke tubers at different initial ethanol concentrations. UCD (FST) refers to University of California, Davis, Food Science and Technology Department, where the *K. marxianus* culture was originally developed.

**MATERIALS AND METHODS**

The method of extracting juice from Jerusalem artichoke tubers was described by Margaritis et al. (16) and Margaritis and Bajpai (17, 18).

*Microorganism and culture media.* The strain of *K. marxianus* UCD (FST) 55-82 used in this study was kindly provided by A. Lachance, Plant Sciences Department, The University of Western Ontario. The culture was maintained on yeast extract-glucose agar slants and was subcultured at weekly intervals. Fermentation medium was comprised of Jerusalem artichoke extract containing 10% total sugars and supplemented with 0.05% Tween 80, 0.01% oleic acid, and 0.01% corn steep liquor. Different initial concentrations of ethanol ranging from 20 to 80 g/liter were added to the fermentation medium before inoculation.
During fermentation the effect of ethanol inhibition on biomass production, sugar utilization, and ethanol production was studied. A fermentation without initial ethanol was run as a control. The pH of the medium was adjusted to 4.6 with 1 N HCl. For the preparation of the inoculum, the yeast was first inoculated into fresh stock medium which consisted of Jerusalem artichoke extract. The stock culture was obtained from a washed slant and then transferred to 50 ml of the medium contained in 250-ml stoppered flasks. After incubation on a New Brunswick rotary shaker at 35°C and 110 rpm for about 24 h, the inoculum was transferred aseptically to 400 ml of the fermentation medium contained in 1-liter Bellco jar fermentors. All suspensions were mixed in the fermentors with magnetic bar stirrers at 200 rpm, and the fermentation temperature was controlled at 35°C. The initial pH for all fermentations was adjusted to 4.6 with 1 N HCl, and it remained almost the same during fermentation.

Analytical methods. During the course of batch fermentations, samples were taken at regular intervals and analyzed for ethanol, total sugars, and cell concentration. Ethanol was analyzed by gas chromatography (16), total sugar was determined by the anthrone reagent method (22), and cell concentration was determined by dry weight (16).

RESULTS AND DISCUSSION

The results for biomass, sugar utilization, and net ethanol production at different initial ethanol concentrations are shown in Fig. 1, 2, and 3. Figure 1 is a semilog plot of the biomass concentrations of K. marxianus cells during batch fermentation in Jerusalem artichoke juice. There

**FIG. 1.** Biomass concentration of *K. marxianus* cells grown in Jerusalem artichoke extract at different initial ethanol (ETOH) concentrations.

**FIG. 2.** Total sugar concentration during batch growth of *K. marxianus* cells at different initial ethanol (ETOH) concentrations.

**FIG. 3.** Net ethanol production by *K. marxianus* cells grown on Jerusalem artichoke extract at different initial ethanol (ETOH) concentrations.
was a decrease in the maximum specific growth rate ($\mu_{\text{max}}$) as the initial ethanol concentration increased from 0 to 80 g/liter. For example, at zero initial ethanol concentration $\mu_{\text{max}}$ was found to be 0.42 h$^{-1}$, while at 80 g/liter the $\mu_{\text{max}}$ was 0.09 h$^{-1}$, which was about 4.7 times less than the control value. Maximum biomass concentration was found to decrease with increasing initial ethanol concentration. Figure 2 shows a semilog plot of total sugar concentration as a function of time at different initial ethanol concentrations. The rate of sugar utilization and the final residual sugars were affected by the initial ethanol concentration. Residual sugar concentrations of 9, 12, 14, 17, and 19 g/liter were found, corresponding to initial ethanol concentrations of 0, 20, 40, 60, and 80 g/liter, respectively. Figure 3 is a semilog plot of the net ethanol concentration produced by K. marxianus cells at different initial ethanol concentrations. It must be noted that the total ethanol concentration to which the K. marxianus cells are exposed during the course of fermentation was the initial amount added to the broth plus the additional ethanol produced (Fig. 3). Both the rates of ethanol production and the final ethanol concentration were affected by the initial ethanol concentration. For example, at initial ethanol concentrations of zero and 80 g per liter the net ethanol concentration was found to decrease from 45 to 35 g/liter, respectively.

The results (Fig. 1, 2, and 3) indicated that the ethanol had an inhibitory effect on cell growth, sugar uptake rate, and ethanol production by K. marxianus cells grown in the inulin-type sugars derived from the tubers of the Jerusalem artichoke. According to Brown (4), the general effect of ethanol is most strongly observed on the cell membrane. Intracellular ethanol concentrations considerably higher than those found in the bulk solution outside the cells have been reported (21). It has been suggested that the actual inhibition appears to be both a general dehydration effect and a specific inhibition of some enzymes, notably alcohol dehydrogenase and hexokinase (4, 9).

The data (Fig. 1, 2, and 3) were used to calculate the biomass yield, ethanol yield, and percentage of original sugars utilized (Fig. 4). The values for percent substrate utilization, ethanol yield, and biomass yield were found to decrease slightly from 90 to 85%, 0.46 to 0.43 g of ethanol per g of sugars utilized, and 0.04 to 0.033 g of cells (dry weight) per g of sugars utilized, respectively, when the initial ethanol concentration was increased from 0 to 80 g/liter. Ghose and Tyagi (11), working with another yeast (Saccharomyces cerevisiae NRRL-Y-132) and using cellulose hydrolysate sugars, found that the ethanol yield ($Y_{\text{PS}}$) and biomass yield ($Y_{X_S}$) remained almost constant at about 0.43 g of ethanol per g of sugars utilized and 0.12 g of cells (dry weight) per g of sugars utilized, respectively, as the ethanol concentration was increased from 0 to 50 g/liter.

Figure 5 shows the maximum specific growth rate ($\mu_{\text{max}}$), calculated from the data shown in Fig. 1 expressed as a function of initial ethanol concentration. To a first approximation, a linear relationship exists between $\mu_{\text{max}}$ and $P_0$, the

![FIG. 4. Total sugar utilization and ethanol and biomass yields of K. marxianus cells as a function of initial ethanol concentration.](http://aem.asm.org)
initial ethanol concentration. A similar linear relationship between \( \mu_{\text{max}} \) and \( P_0 \) has been reported by Ghose and Tyagi (11), working with \( S. \) cerevisiae, and by Hinshelwood (13) for \( B. \) lactis aerogenes. Holzberg et al. (14) reported for a yeast strain that a minimum ethanol concentration existed below which there was no inhibition and above which there was a linear relationship between \( \mu_{\text{max}} \) and ethanol concentration. The data shown in Fig. 5 may be correlated according to equation 1, \( \mu_{\text{max}} = \mu_0 \left[ 1 - \frac{P_0}{P_m} \right] \), where \( \mu_{\text{max}} \) is the maximum specific growth rate per hour at any \( P_0 \), \( \mu_0 \) is the maximum specific growth rate per hour at zero initial ethanol concentration, \( P_0 \) is the initial ethanol concentration in grams per liter, and \( P_m \) is the maximum initial ethanol concentration above which \( \mu_{\text{max}} \) equals 0 g/liter.

Extrapolation of the data shown in Fig. 5 gave \( P_m = 95 \) g of ethanol per liter and the experimental value of \( \mu_0 = 0.42 \) h\(^{-1} \). Ghose and Tyagi (11), working with \( S. \) cerevisiae cells grown in cellulose hydrolysate, found a \( P_m \) of 87 g of ethanol per liter, at which \( \mu_{\text{max}} = 0 \). Aiba et al. (1), working with a yeast, found no cell growth for ethanol concentrations higher than 76 g/liter whereas Bazua and Wilke (3) found that \( S. \) cerevisiae did not grow or produce ethanol at an ethanol concentration higher than 93 g/liter.

The logarithmic growth phase results (Fig. 1 and 3) were computer fit with a correlation higher than 0.992, and the corresponding time derivatives, \( dP/dt \) and \( dx/dt \), at specified time intervals were obtained. Figure 6 is a plot of corresponding \( dP/dt \) against \( dx/dt \) values to check whether ethanol was a growth-associated product. For growth-associated product fermentation equation 2 applies (2): \( dP/dt = \alpha \left( \frac{dx}{dt} \right) \), where \( dP/dt \) is the rate of ethanol production (grams of ethanol per liter per hour), \( dx/dt \) is the rate of biomass production (grams of cells [dry weight] per liter per hour), and \( \alpha \) is the growth-associated constant (dimensionless). As shown in Fig. 6 there was a linear relationship to a first approximation, and the slopes of these lines were affected by the initial ethanol concentration in the broth. Figure 7 shows the relationship between \( \alpha \) and initial ethanol concentration.

The results presented in this paper show that the ethanol tolerance level of \( K. \) marxianus UCD (FST) 55-82 cells grown in the inulin-type sugars derived from the Jerusalem artichoke is

![FIG. 5. Maximum specific growth rate of \( K. \) marxianus cells as a function of initial ethanol concentration.](image)

![FIG. 6. Growth-associated plot for \( K. \) marxianus cells at different initial ethanol (ETOH) concentrations.](image)

![FIG. 7. Growth-associated constant (\( \alpha \)) as a function of initial ethanol concentration.](image)
about the same as the ethanol tolerance level reported in the literature for different *S. cerevisiae* strains. Mutation work is underway in our laboratory with a large number of *K. marxianus* strains to increase the ethanol tolerance level reported in this paper.

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LITERATURE CITED