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Kinetics studies of product inhibition in alcoholic fermentation

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This paper presents inhibitory effect of ethanol on growth of cells and ethanol production. A linear fall was observed of survival (%) of *Kluyveromyces Thermotolerans* cells with time. Change in death rate constant of cells beyond certain range of ethanol concentration was due to shift in mechanism of cells inhibition. Pronounced inhibitory effect was observed at higher ethanol concentrations in broth. Decrease in optimum temperature for growth of cells was found with added ethanol concentration. This also confirms more inhibitory effect of product on microorganism at higher temperature. This study confirms both reversible and irreversible inhibition of cells by ethanol.

Keywords: Alcoholic fermentation, Product inhibition, Thermo tolerant yeast, Inhibitory effect

Introduction

Ethanol as fermentation product is used in many aspects of human life, including consumption of alcohol beverages and use of ethanol-based biofuels[1]. Fermentation activity of ethanol producing organisms declines progressively as ethanol concentration increases in medium[2]. Mechanisms[3] have been proposed for inhibitory effects of ethanol. Inhibition of cell growth and viability was observed to increase with increasing ethanol concentrations, whereas high fermentative capacity was only inhibited at high ethanol concentrations[3-5]. Relationship between ethanol concentration and specific growth rate suggested a strong inhibitory effect of ethanol on specific culture growth rate[6]. Majority of kinetic models, describing microbial growth during ethanol fermentation, use a formal macroapproach to bioprocessing[7]. Such models are empirical and based on Monod's equation[8]. In many models, effect of ethanol inhibition is explained via mechanism of non-competitive inhibition of a simple reversible enzymatic reaction[9,10]. In non-mechanistic approach, it is assumed that inhibition by ethanol follows a linear[11,12], exponential[13,14], hyperbolic or another non-linear formula[15,16]. Inhibitory effect of ethanol[17] has been studied both on yeast growth and on fermentation for a continuous process of alcoholic fermentation in a tower reactor with recycling of flocculating cells.

Most widely employed method for determining ethanol tolerance involves suppression of cell growth in presence of exogenous ethanol[18]. This is primarily because tolerance to ethanol is not influenced by nutritional conditions or growth states of cells[19]. Furthermore, values obtained correlate well with upper limits of ethanol production reported in sake fermentations[20]. Jimenez & Van-Uden[21] proposed a method based on extracellular acidification for rapid testing of ethanol tolerance in yeast. In yeast, ethanol has an effect on many different cellular behaviors and processes related to cell death, including stress responses[22], changes in membrane fluidity[23], protein structure[24] and mRNA export from nucleus[25]. Ethanol-induced cell death exhibits features in common with apoptosis[26]. Palmqvist *et al*[27] discussed generation of inhibitors during degradation of lignocellulosic materials, and effect of these on fermentation yield and productivity. Sa-Correia[28] suggested that in addition to ethanol enhanced thermal death, another form of ethanol-induced death occurs. Lucas[29] studied temperature profile of growth thermal death and ethanol tolerance of xylose fermenting yeast (*Candida shehatae*) and showed that toxic effects of ethanol on either side of temperature

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plateau, depressing maximum temperature for growth and increasing minimum temperature for growth. Leao[30] reported temperature relations of glucose fermentation with and without ethanol by resting cells over temperature range. Van Uden[5] suggested that target of thermal death and of ethanol enhanced thermal death in *Saccharomyces cerevisiae* is macromolecular site located in inner mitichondrial membrane. Target of ethanol induced death that predominates at low and intermediates growth temperatures is located in plasma membrane. Sa-Correia [31] reported that ethanol depressed maximum temperature for growth and enhanced thermal death in *S cerevisiae* and *Kluyveromyces fraglis*.

This study presents kinetics of inhibition of product on cell viability and specific growth rates with an objective to investigate mechanism of product inhibition.

Experimental Section

Microorganism

A pure culture of Kluyveromyces thermotolerans MTCC 30, obtained from Institute of Microbial Technology, Chandigarh, India, was used. K. thermotolerans was maintained on MYGP slants and stored at 4°C in a refrigerator. Stock culture was sub cultured once in a month to maintain potency and metabolic activities of culture.

Seed Medium

Cells of *K. thermotolerans* were grown in Erlenmeyer flask (500 ml) containing 100 ml of seed medium (composition: malt extract, 3.0; yeast extract, 3.0; peptone, 5.0; and glucose 100 g/l) with pH 5.0 and temperature at 40°C. Media (10 ml) was taken in each test tube and after putting cotton plug and aluminum foil, test tubes were sterilized at 15 psi for 15 min. Test tubes were put on slant platform for solidification. Slants thus obtained were used for sub culturing of microorganism. *K. thermotolerans* was inoculated in each test tube using inoculation needle in laminar flow, already sterilized by UV-light. Finally, it was kept in incubator for 48 h.

Biomass Assay

For analysis, 10 ml samples were drawn and 1 ml of 32% formaldehyde solution was added to stop cells growth. Cells were centrifuged at 4000 rpm and supernatant was stored in refrigerator to measure reducing sugar and ethanol. Cell residues after washing with distilled water were suspended in 0.85% NaCl solution. Optical density of cell suspension was measured at 525 nm by UV-VIS spectrophotometer using distilled water as a blank. Dry weights were determined by transferring cell residues to a preweighed aluminum foil cup. It was dried at 90°C overnight in an oven. Dry cell mass was determined by subtracting weight of empty aluminum foil. Viable cell counts were determined by methylene blue staining technique and cell count was determined using Haemocytometer. Ruling on chamber is 1/400 mm^2 and 1/10 mm deep.

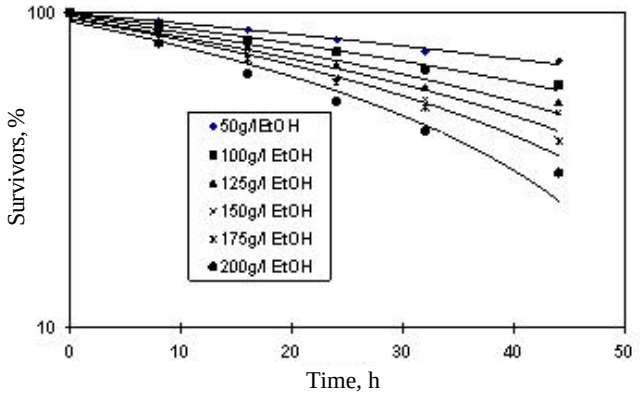

Fig. 1—Experimental data for death rate of *K. thermotolerans* in various concentrations of ethanol

Estimation of Ethanol

Ethanol content was measured by a gas chromatograph (Sigma Instruments, Baroda, India) fitted with a column (diam, 0.65 cm; length, 2 m) packed with porapack-Q (120 mesh). Nitrogen (flow rate, 40 ml/min) was used as carrier gas. Flame ionization detector (FID) was maintained at 150°C and oven was put on 4 h in advance before stabilizing temperature. Injector temperature was set at 160°C, while column operated isothermally at 150°C. After stabilizing temperature, 1 µl of samples were injected through a rubber septum with a micro liter syringe.

Results and Discussion

Effect of Ethanol Concentration on Cell Viability

Separate experiments were performed taking ethanol (conc. 50, 100, 125, 150, 175 and 200 g/l) in Erlenmeyer flasks maintained under strict anaerobic conditions. Cells (4.5 g/l) were added in each flask and incubated at 40°C. At different time intervals samples were taken and analyzed. Survivors (%) were calculated by colony counts in petri plates and plotted against time with ethanol concentration (Fig. 1). Data were found consistent with a first-order decay rate of viable population. Computed decay rate constants of cells (K_d) were plotted against

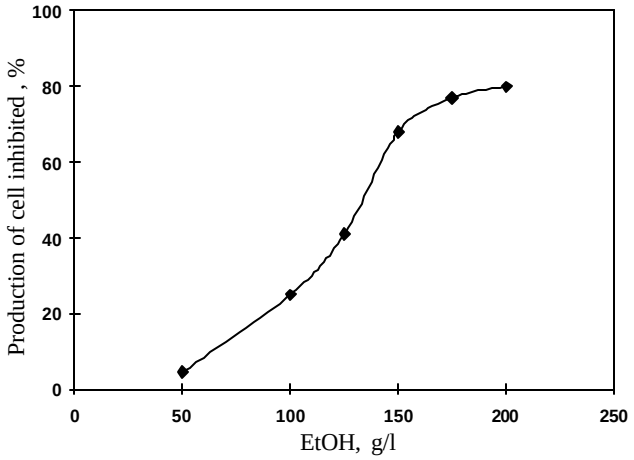

Fig. 2—Product dose response curve of *K. thermotolerans*

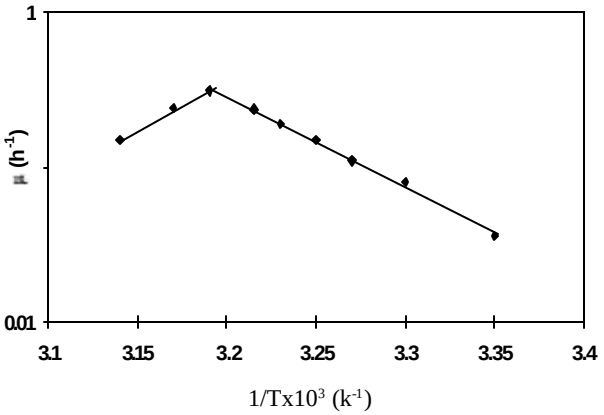

Fig. 3—Arrhenius plots of *K. thermotolerans*: Specific growth rates without initial addition of ethanol

reciprocal of ethanol concentration, which shows a sudden change in the slope at ethanol concentration of 133 g/l, might be a shift in mechanism of destruction of cells[32,33]. It can be simply expressed as

$$\log K_d = (-p/2.303\, C_e) + \log A' \quad \ldots(1)$$

$$K_d = A'\, e^{-p/Ce} \quad \ldots(2)$$

where, K_d is decay rate constant of cells and is a function of product concentration temperature, A' is frequency factor of cell inhibition, C_e is concentration of product, and p is constant (dependent on product concentration).

Product Dose Response on Cell Growth

Under effect of ethanol concentration on inhibition of growth of *K. thermotolerans*, proportion of viable cells decreased with increases in C_e (Fig. 2), which can be expressed as

$$C_e = K\,(Y/1\text{-}Y)^{1/n} \quad \ldots(3)$$

where Y, proportion of cells inhibited; n, index of inhibition degree; and K, inhibition coefficient.

Asymptotic dose response curve (Fig. 2) indicates that at 133 g/l ethanol, 50% of viable cells were inhibited. Constants (n and K) can be determined from logarithm of Eq. (3) as

$$\log C_e = 1/n \log (Y/1\text{-}Y) + \log K \quad \ldots(4)$$

A plot of $\log C_e$ vs $\log [Y/(1\text{-}Y)]$ is linear. From slope and intercept of this line, n (3.45) and K (130 g/l) were determined with respect to product.

Influence of Initial Ethanol Concentration on Specific Growth Rate at Different Temperatures

Three sets of cultivation experiments were carried out at each temperature between 30-45°C. In medium, no ethanol was added. From data of cell growth (X) vs cultivation time (t), values of μ_0 (specific growth rate with no added ethanol) were determined. From plot of μ_0 vs corresponding reciprocal of absolute temperature (Fig. 3), activation energy (E) for cell growth was computed. At low ethanol concentrations (50-100 g/l) in the medium, viable cells (75-80%) were found present until the end of cultivation. However, in presence of high ethanol concentration, cell viability decreased to 15-20% levels. Values of μ_i (specific growth rate in presence of i g/l added ethanol) were plotted (Fig. 4) against corresponding reciprocal of absolute temperature to determine values of E. When E was plotted against ethanol concentration in the medium, slope of E profile changed sharply at ethanol (> 125 g/l), and at ethanol (> 150 g/l), E was almost constant and cell viability was very low, might be due to dissolution of membrane lipids and increased membrane pore size, thereby increasing cell membrane permeability of exterior ethanol inside cell[33]. Ultimately, it produced a higher lethal effect for inhibition. On the other hand, since below 125 g/l product concentration, E increased linearly with product concentration, and cell viability was 75-85%, phenomenon can be explained by suppression of cells due to repression of some of its enzymes. This plot also indicated that as added ethanol increased in broth, optimum temperature for growth of *K. thermotolerans* decreased. At different added ethanol concentrations (g/l), optimum growth temperatures (°C) were: 0, 40; 50, 40; 100, 38; 125, 36; 150, 34; 175, 32; and 200, 30. This indicates more

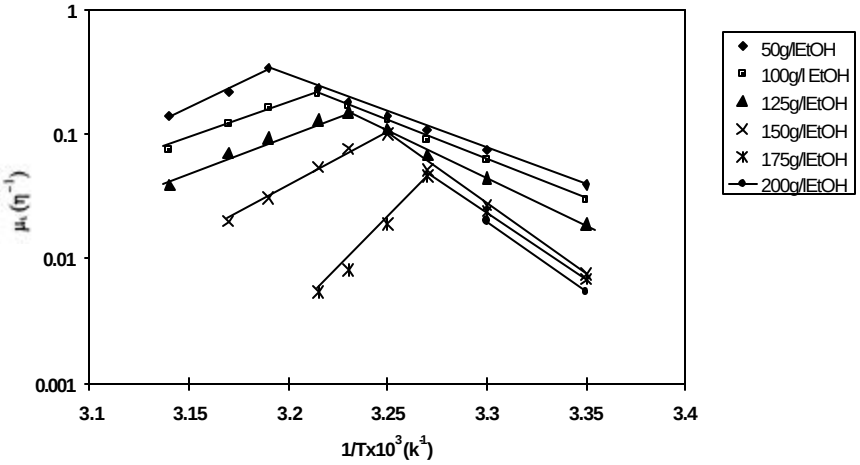

Fig.4—Arrhenius plots of *K. thermotolerans*: Specific growth rates in the presence of various concentration of ethanol

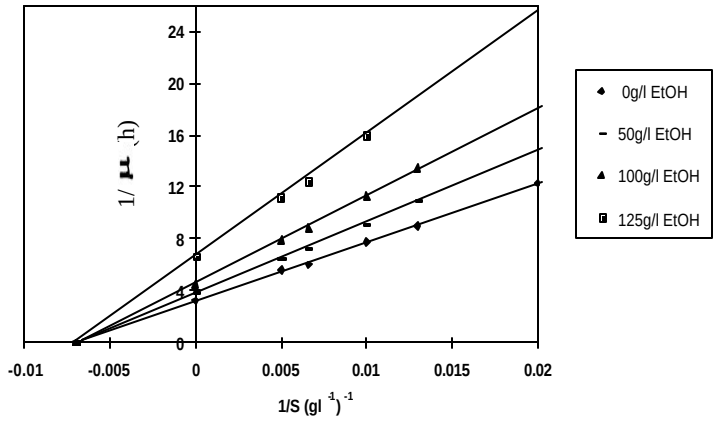

Fig. 5—Lineweaver-Burk Plot for 1/μ vs 1/S with various ethanol concentrations at 40°C

inhibitory effects of products on organism at higher temperatures compared to lower temperatures.

Dependence of Inhibition on Ethanol Concentration and Temperature

Experiments were carried out at several temperatures (30-40°C) taking separate ethanol concentrations (50-200 g/l). From profile of X vs cultivation time, μ_i were calculated. Values of $1/\mu$ were plotted against $1/S$ (obtained from data of X and S vs cultivation time at 40°C) with ethanol concentrations. It was observed that inhibition by ethanol on *K. thermotolerans* is of noncompetitive type (Fig. 5), which is quite similar to reported[34] studies, wherein effect of product concentration on μ values were confirmed by Lineweaver - Burk plot, indicating non-competitive inhibition. Similar behavior is reported in earlier studies[2,33-36]. At 40°C, cells did not grow above 125 g/l ethanol levels and hence, Lineweaver - Burk plot was made considering levels up to 125 g/l of ethanol concentration as parameter. Values of the fraction of inhibition (I=1-μ_i/μ_o) were calculated at various levels of added ethanol concentrations in medium. Double reciprocal plot between I and C_e (Fig. 6) indicated existence of a critical inhibitory concentration of product, below which relation between I and C_e follows Eq. (5) proposed by Webb[37].

$$1/I = 1 + K_i /C_e \qquad \ldots(5)$$

where, K_i is inhibition constant of cell growth.

Above these critical concentrations, however this relation was not followed, possibly because of pseudo-irreversible nature of inhibition. Levels of inhibitory critical concentrations of product decreased

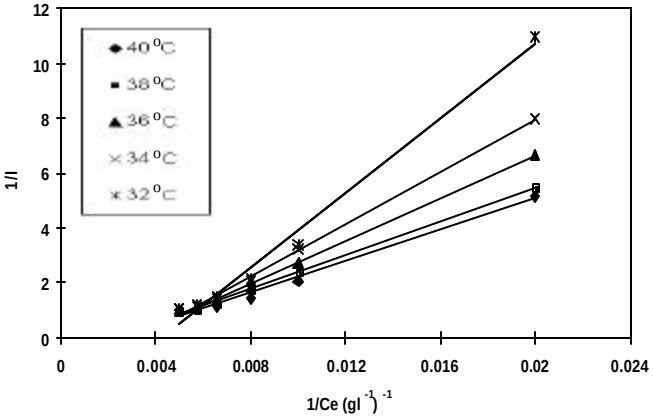

Fig. 6—Effect of temperature on ethanol tolerance on net growth

[225 g/l (30°C) to 150 g/l (40°C)] with an increase in cultivation temperature (Fig. 6). From slopes of Fig. 6, values of K_i at each temperature were determined. Computed values of K_i were then plotted against reciprocal of absolute temperature. This established that K_i increased nonlinearly with inverse of temperature. From slope of this curve, values of enthalpy of cell growth (dHi) were computed, which showed a variation in dHi, due to variation of degree of inhibition cells at different temperatures. Higher working temperatures (Fig. 3) were also responsible for inhibition of ethanol even at low concentration levels.

Conclusions

Inhibitory effect of ethanol on growth of cells and ethanol production indicated that survival (%) of *Kluyveromyces* cells varied between 70% and 31% after 44 h of fermentation using initial concentration 50 g/l and 200 g/l of ethanol, respectively. Above critical concentration of ethanol (133 g/l), drastic decrease in survival might be due to shift in mechanism of cells inhibition, which has been established by product dose response studies. Increased activation energy (E) with higher ethanol concentrations has been observed. Increased ethanol concentration in broth affects inversely the optimum temperature for growth of *Kluyveromyces* sp. This also confirms greater inhibition at higher temperature. Inhibition of cells by ethanol is reversible as well as irreversible up to critical concentration (133 g/l), beyond which it is only irreversible.

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