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Ethanol adaptation in a thermotolerant yeast strain *Kluyveromyces marxianus* IMB3

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The maximum ethanol concentration produced from glucose in defined media at 45°C by the thermotolerant yeast *Kluyveromyces marxianus* IMB3 was 44 g L^{-1}. Acclimatisation of the strain through continuous culture at ethanol concentrations up to 80 g L^{-1}, shifted the maximum ethanol concentration at which growth was observed from 40 g L^{-1} to 70 g L^{-1}. Four isolates were selected from the continuous culture, only one of which produced a significant increase in final ethanol concentration (50 ± 0.4 g L^{-1}), however in subsequent fermentations, following storage on nutrient agar plates, the maximum ethanol concentration was comparable with the original isolate. The maximum specific ethanol production rates (approximately 1.5 g $(gh)^{-1}$) were also comparable with the original strain except for one isolate (0.7 g $(gh)^{-1}$). The specific ethanol productivity decreased with ethanol concentration; this decrease correlated linearly (rval 0.92) with cell viability. Due to the transience of induced ethanol tolerance in the strain it was concluded that this was not a valid method for improving final ethanol concentrations or production rates.

Keywords: adaptation; ethanol tolerance; specific ethanol production; viability

Introduction

The effects of ethanol on yeast have been investigated extensively. From a recent review [10], these were summarised as: damage to the plasma membrane as well as the membranes of various cell organelles; denaturation of cellular proteins; inhibition of transport systems; and a shift in temperature-growth profile. This can result in a loss of cell viability, reduction in specific growth rate and reduction in specific fermentation rate. Each of these parameters may not be affected to the same extent, which has led to differences in the measurement and definition of ethanol tolerance. Many different methods of measuring ethanol tolerance have been reported in the literature including ethanol-induced leakage of UV-absorbing substances [18], extracellular acidification [13,17], as well as the use of endogenous and/or exogenously supplied ethanol to attain a certain inhibitor concentration and measuring viability, fermentation and/or growth rates. Furthermore, the ethanol tolerance of an organism is variable and can be influenced by osmotic activity [9,18], nutrient limitation [11], media composition [12], temperature and previous exposure to ethanol [1,3,19] with certain media components eg trehalose [14], and metal cations Ca^{2+}, Mg^{2+}, Zn^{2+}, K^{+} [6,8,12,20,21] having particularly pronounced effects.

Workers at the University of Ulster, Coleraine have isolated a thermotolerant yeast from soil samples collected at Associated Distilleries, India. The strain, designated *Kluyveromyces marxianus* var *marxianus* IMB3, is capable of growth and fermentation at temperatures of 25–50°C [2]. *K. marxianus* has been defined as a respiratory yeast [4], thus only low amounts of glucose are converted to ethanol under aerobic conditions and high specific ethanol production rates can only be achieved under anaerobic or oxygen-limited conditions. This novel thermotolerant yeast strain has potential for use in industrial fermentations in hot climates where the lack of control of fermentation temperatures can lead to cessation of production for up to 3 months of the year (personal communication, Associated Distilleries, India). The maximum ethanol concentration produced by the strain at 45°C in defined media was 44.3 ± 2.5 g L^{-1}. This was low when compared to strains of *Saccharomyces cerevisiae* or *Scizosaccharomyces pombe* which are commonly used for ethanol production and can produce up to 95 g L^{-1} ethanol [5]. Many authors report links between tolerance to ethanol and temperature in yeast. *Saccharomyces cerevisiae* has a single integral plasma membrane heat shock protein (Hsp), inducible by several stresses, including heat shock and ethanol exposure [16]. Chen and Piper [7] however, showed that overexpression of ubiquitin (a protein expressed under a variety of stress conditions) slightly increased tolerance to ethanol and osmostic activity whilst thermotolerance was unaffected. Strain IMB3 has improved thermotolerance compared to other strains of *K. marxianus* [2]. This thermotolerance may have been induced by the hot temperatures prevalent at the site where the strain was isolated, thus similar improvements in ethanol tolerance may be induced through culturing the organism at increasing ethanol concentrations.

Materials and methods

Microorganism, maintenance and preparation of inoculum

Kluyveromyces marxianus var *marxianus* was obtained from soil samples collected from the ground at Associated Distilleries, India. They were selected by enrichment cul-

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ture and maintained on nutrient agar slopes at 4°C [2]. The isolate designated *Kluyveromyces marxianus* IMB3 is deposited with NYCC (Institute Food Research, Norwich, UK). Inocula were prepared in 100-ml (or 250-ml) shake flasks containing 50 ml (100 ml) of yeast fermentation media (MYFM) [2], and used to inoculate a New Brunswick chemostat with a working volume of 0.6 L, (50 ml of inoculum) for continuous culture or a Braun Biostat-B fermenter, working volume 5 L, (100 ml of inoculum) for batch fermentations.

Selection under continuous culture

A New Brunswick chemostat was filled with MYFM media (0.6 L), pH was controlled automatically to 5.0 using potassium hydroxide (2 M) and sulphuric acid (10%). Glucose solution was sterilised separately and added to the chemostat prior to inoculation giving a resultant glucose concentration of 120 g L^{-1}. Agitation was maintained at 150 rpm and the air flow rate was 1 L min^{-1}. After 12 h of batch operation a 10-L carboy containing MYFM plus glucose (120 g L^{-1}) was connected to the fermenter, and the fresh media were delivered to the vessel using a Watson-Marlow 502S pump. Ethanol, glucose and biomass concentrations were recorded, and once a steady state was established ethanol was added to the MYFM media to give a concentration of 8 g L^{-1} in the chemostat. After approximately 12 h a new steady state, ie ethanol, biomass and glucose concentrations remaining constant, was observed. The ethanol concentration in the feed was increased after each steady state was reached, simultaneously the dilution rate was adjusted to prevent wash-out. Aseptic samples were taken from the fermenter at ethanol concentrations of 45–70 g L^{-1} and tested in batch fermentations.

Batch fermentation

Fermentations were carried out at 45°C and pH 5.0 in a 5-L Braun Biostat-B fermenter containing 4 L of MYFM (120 g L^{-1} glucose). The fermenter was inoculated with 100 ml of the shake flask culture. The pH and temperature were monitored and controlled using Braun software. During the aerobic phase agitation was maintained at 300 rpm and the air flow rate was 4 L min^{-1}. After approximately 24 h the air flow was switched off and the agitation was reduced to 150 rpm. Biomass (absorbance at 660 nm of diluted broth samples using a standard curve) and ethanol concentrations (clarified broth samples analysed using a Perkin-Elmer capillary gas chromatograph [2]) were measured periodically to determine the specific ethanol productivity. Samples were also removed for determination of cell viability; following serial dilution, triplicate 0.1-ml samples of each diluted sample were spread onto nutrient agar plates and incubated at 45°C for 48 h, and the number of colonies counted.

The specific growth rate of the original isolate was determined at various ethanol concentrations by adding ethanol to the MYFM media prior to inoculation, and measuring subsequent changes in biomass concentration.

Results and discussion

The maximum ethanol concentration produced from glucose in defined media at 45°C by the thermotolerant yeast

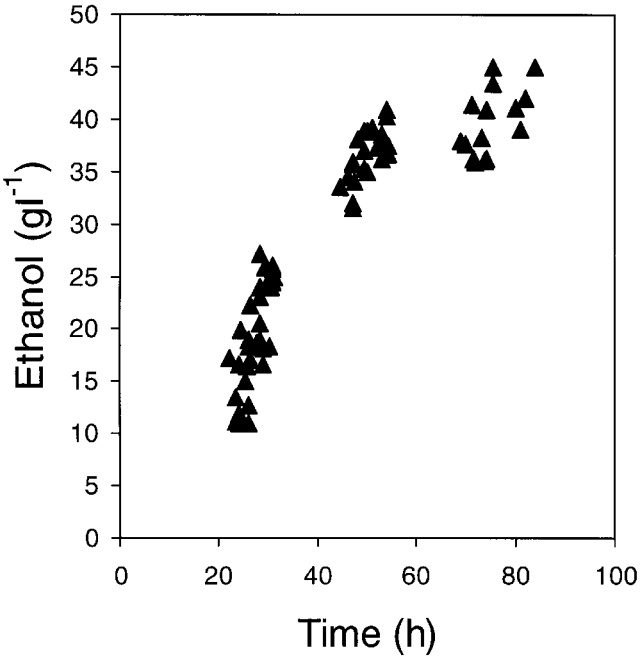

Figure 1 Ethanol production during batch fermentation of *K. marxianus* IMB3. Results are from eight fermentation runs.

Kluyveromyces marxianus IMB3 in batch culture was 44.3 ± 2.5 g L^{-1} (Figure 1). The specific ethanol production rate (qPpX) decreased with ethanol concentration, with 50% of activity lost at an ethanol concentration of approximately 15 g L^{-1} ethanol (Figure 2).

$$\text{qPpX} = \frac{\text{g ethanol produced}}{\text{g cell dry mass} * \text{time}} \quad (1)$$

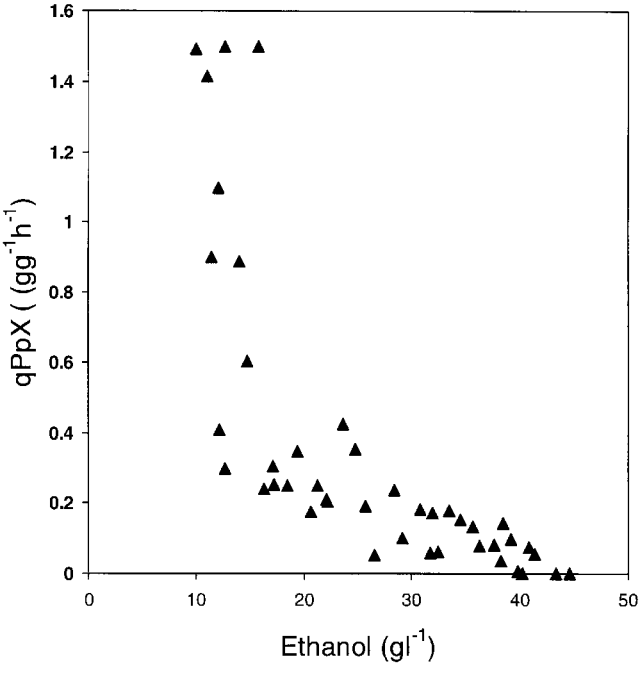

Figure 2 Specific ethanol production during batch fermentation of *K. marxianus* IMB3. Results are from eight fermentation runs.

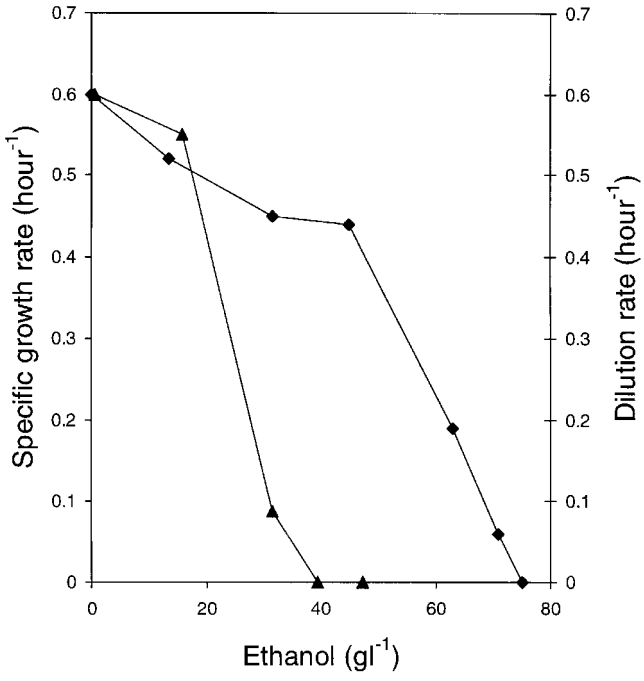

Figure 3 Maximum specific growth rate of original *K. marxianus* IMB3 during batch fermentation (▲) and dilution rates (◆) during selection of isolates in continuous culture. Growth medium: MYFM 120 g L^{-1} glucose and ethanol.

The maximum specific growth rate of the original culture during batch fermentation, in media containing exogenous ethanol was compared with the dilution rate achieved in continuous culture, where the ethanol concentration (a combination of endogenous and exogenous ethanol) was gradually increased over a period of weeks (Figure 3). The original IMB3 would not grow when inoculated into a batch culture containing 40 g L^{-1} of ethanol, although it did remain viable for up to 48 h at these conditions, ie it could be recovered by either lowering the temperature or by streaking onto nutrient agar plates. At 47 g L^{-1} ethanol, the viability of the inoculum was lost within 1 h of inoculation. The inhibitory effect of ethanol on growth, fermentation and viability is summarised in Table 1.

The maximum ethanol concentration at which growth was observed, shifted from 40 g L^{-1} in batch culture to 70 g L^{-1} in the chemostat. In the batch experiments cells were in the lag phase when exposed to the ethanol, whilst in the continuous experiments the ethanol was added during exponential growth. Norton *et al* [15] demonstrated that the rate of cell growth during exposure to exogenously added

Table 1 Summary of the inhibitory effects of ethanol on growth, fermentation and viability

Maximum ethanol concentration for growth in batch culture (exogenous)	Maximum ethanol concentration produced in batch culture	Maximum ethanol concentration for viability
32 g L^{-1}	44.3 ± 2.5 g L^{-1}	40 g L^{-1} (endogenous) 43 g L^{-1} (exogenous)

Table 2 Maximum ethanol concentration produced in batch culture on defined media at 45°C by original *K. marxianus* IMB3 plus four isolates selected from continuous culture on media containing ethanol

Isolate	Ethanol concentration at isolation (g L^{-1})	Final ethanol concentration (g L^{-1})	qPpX (max) (g g^{-1} h^{-1})
Original	–	44.3 ± 2.5	1.5
I	70	39.05 ± 0.15	0.7±
II	63	42.06 ± 0.8	1.5
III	47	45.0 ± 0.33	1.5
IV	40	50 ± 0.4	1.5

ethanol is a significant factor in the resistance of yeast to high ethanol concentrations, however their results demonstrated higher survival rates in slower growing cells. It was, therefore, believed that the cells were acclimatising to higher ethanol concentrations.

Four isolates were selected from media with ethanol concentrations of 40–70 g L^{-1}. Table 2 shows the final ethanol concentrations produced by the selected isolates in batch culture, and the maximum specific ethanol production rates. No significant difference was observed between the final ethanol concentration of three of the isolates and the original strain. Isolate IV showed a slight improvement in final ethanol concentration (50 g L^{-1} ± 0.4) (Figure 4). Given the similarity between the exogenous ethanol concentration at which cells remained viable (43 g L^{-1}) and the maximum ethanol concentration produced in batch culture (44 g L^{-1}), this result was disappointing. The maximum specific ethanol production rates of isolates II, III and IV were also similar to the original strain at approximately 1.5 g $(gh)^{-1}$. Isolate I (selected from media containing 70 g L^{-1} ethanol) had a significantly lower specific ethanol production rate at

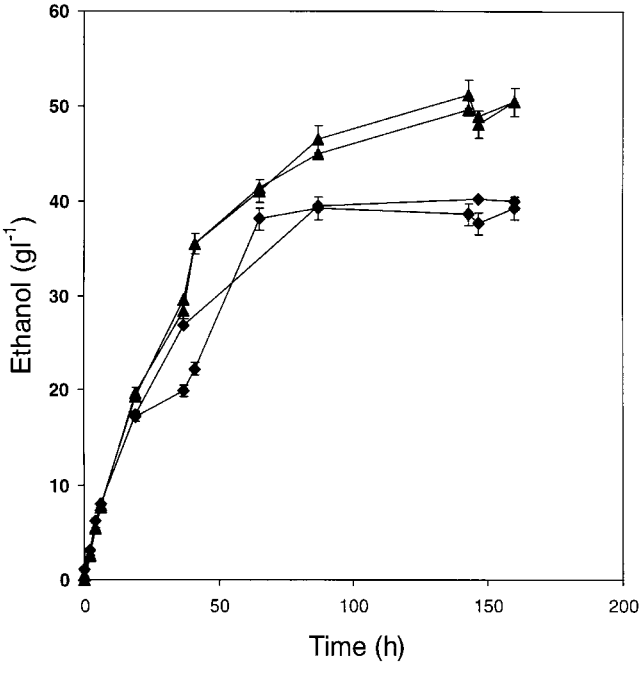

Figure 4 Ethanol production during batch fermentation of original *K. marxianus* IMB3 (◆) and Isolate IV (▲).

0.7 g (gh)$^{-1}$, which represented a 50% reduction compared to the original isolate. This result has implications for continuous ethanol processes or batch fermentation followed by cell recycle, a method used extensively by the ethanol industry in Brazil (personal communication, HV Amorin, Fermentec s/c Itda, Brazil). Strains remaining viable at the end of the fermentation process will be preferentially selected, although they may have a lower specific ethanol production rate than the original inocula. The maximum specific ethanol production rate of Isolate IV did not alter, however the specific activity profile shifted, so higher production rates were observed at a given ethanol concentration (Figure 5). Isolate IV was stored on nutrient agar plates and used in subsequent fermentations, during which the maximum ethanol concentration produced fell to similar values as the original strain.

Utilisation of cell dry mass measurements to determine qPpX (Eqn 1) does not differentiate between viable and non-viable cells. Viable cell numbers (Vc) during batch fermentation decreased linearly with specific ethanol productivity (Figure 6).

$$qPpX = 0.95 * Vc + 0.025 \quad (2)$$

Thus ethanol inhibits fermentation capacity and viability to the same extent. This result agrees with the initial studies which showed close correlations between final ethanol concentrations produced in batch fermentations (44 g L^{-1}) and the ethanol concentration at which cells remained viable (43 g L^{-1}).

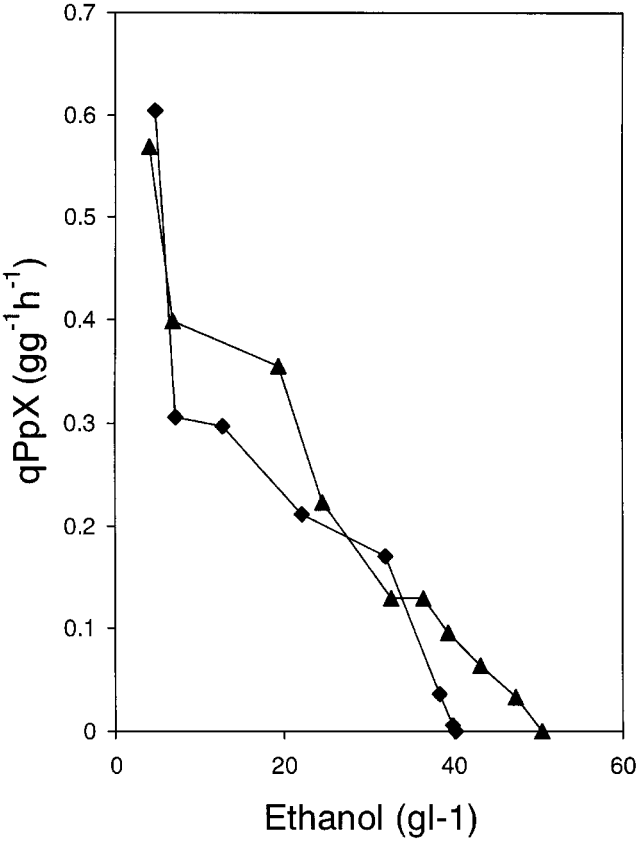

Figure 5 Specific ethanol production rate of original *K. marxianus* IMB3 (◆) and Isolate IV (▲) during batch fermentation.

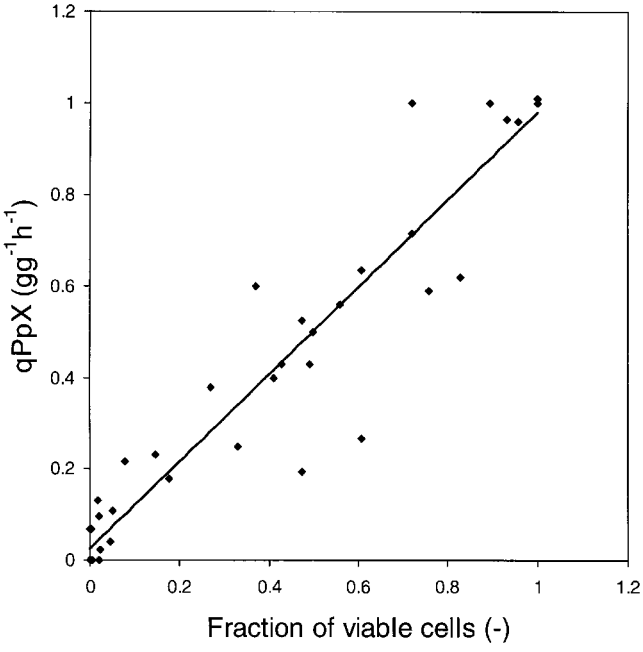

Figure 6 Correlation between apparent specific ethanol productivity (on a cell dry weight basis) and fraction of viable cells remaining, during batch fermentation.

Previous studies have reported differences between exogenous and endogenous ethanol on kinetic performance [11], however, viability of IMB3 was lost at between 40–47 g L^{-1} of exogenous ethanol and at 43 g L^{-1} of endogenous ethanol, demonstrating that both exogenous and endogenous ethanol had a similar inhibitory effect.

Studies with *Saccharomyces cerevisiae* have demonstrated various responses to ethanol stress including the induction of 'heat-shock' proteins, increases in intracellular trehalose and changes in the lipid composition of the plasma membrane, and that these responses can be correlated to improved ethanol tolerance [1]. The results from this work have shown that growth in the presence of ethanol led to improved tolerance of ethanol of the low ethanol-tolerant strain *Kluyveromyces marxianus* IMB3, when defined as the ability to remain viable in the presence of exogenously added ethanol. However, this ability to remain viable at increased ethanol concentrations did not necessarily lead to the ability to produce ethanol at these conditions. Isolate IV which did remain productive at higher ethanol concentrations, demonstrated the transient nature of the response.

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