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Characterisation of a novel thermotolerant yeast, *Kluyveromyces marxianus* var *marxianus:* development of an ethanol fermentation process

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The fermentation characteristics of the novel, thermotolerant, isolate *Kluyveromyces marxianus* var *marxianus* were determined to evaluate its aptitude for use in an ethanol production process. Sustainable growth was not observed under anaerobic conditions, even in the presence of unsaturated fatty acid and sterol. A maximum ethanol concentration of 40 g L^{-1} was produced at 45°C, with an initial specific ethanol production rate of 1.7 g g^{-1} h^{-1}. This was observed at ethanol concentrations below 8 g L^{-1} and under oxygen-limited conditions. The low ethanol tolerance and low growth under oxygen-limited conditions required for ethanol production implied that a simple continuous process was not feasible with this yeast strain. Improved productivity was achieved through recycling biomass into the fermenter, indicating that utilising an effective cell retention method such as cell recycle or immobilisation, could lead to the development of a viable industrial process using this novel yeast strain.

Keywords: thermotolerance; process development; novel yeast

Introduction

Fuel ethanol industries were developed in many countries as a response to the world oil crisis of 1973 and 1975 [9,18,19]. It was believed that domestic ethanol production for use as a transport fuel extender would have environmental, social and economic benefits through a reduction in noxious emissions, utilising surplus agricultural activity and reducing dependence on fossil fuels. Numerous reports now conclude that the production of fuel ethanol does not achieve these objectives [8,12,14] due to the high production costs of ethanol ($0.64 per L [15]) compared with fossil fuels ($0.30 per L for gasoline). Qureshi and Manderson [15], suggested that a continuous fermentation process incorporating *in situ* ethanol recovery improved the economics of ethanol production, making it competitive with traditional fossil fuels.

Workers at the University of Ulster have isolated a facultative, thermotolerant yeast designated *Kluyveromyces marxianus* var *marxianus* IMB3 [4,5]. Efficient ethanol production requires rapid fermentation leading to high ethanol concentrations, therefore a yeast strain must have a good specific growth rate and specific ethanol production rate at high osmotic activities and ethanol concentrations. *K. marxianus* has been defined as a respiratory yeast [6], thus only low amounts of glucose are converted to ethanol under aerobic conditions. Many industrial processes use an acid wash step to reduce bacterial contamination, therefore the strain must remain viable at low pH.

To ascertain the potential of this strain of *K. marxianus* for ethanol production, important preliminary fermentation characteristics were determined at 45°C. This paper describes the effect of specific oxygen transfer rate on growth, plus ethanol and glucose concentration on specific growth and ethanol production rates. The results of these studies were used to evaluate various fermentation process options.

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Materials and methods

Microorganism and maintenance

Strains of *Kluyveromyces marxianus* var *marxianus* were isolated from soil samples collected from the ground at Associated Distillery, Northern India. They were selected by enrichment culture and maintained on nutrient agar slopes at 4°C. The isolate designated *Kluyveromyces marxianus* var *marxianus* IMB3 has been deposited with NCYC (Institute of Food Research, Norwich, UK).

Media and inoculum preparation

Inocula were prepared in 100-ml shake flasks containing 50 ml of yeast fermentation medium (MYFM): yeast extract (3 g L^{-1}); peptone (2 g L^{-1}); KH_2PO_4,(2 g L^{-1}); NH_4SO_4, (2 g L^{-1}); $MgSO_4{\cdot}7H_2O$ (1 g L^{-1}) and $MnSO_4{\cdot}H_2O$ (0.1 g L^{-1}). The solution was adjusted to pH 5.0 with KOH. A glucose solution was injected into the sterilised flasks through a Gelman Acrodisc filter. The resulting glucose solution was 10 g L^{-1}. The flasks were inoculated with a single colony of *K. marxianus* from an agar slope and incubated for 12–24 h at 45°C and 200 rpm in a New Brunswick orbital shaker. The flask culture was used to inoculate either a fermenter (5 L) or further shake flasks (250 ml) containing MYFM media.

Shake flask fermentations

Five millilitres of inoculum from a shake flask were used to inoculate a 250-ml flask containing 100 ml or 50 ml of MYFM. The flask was incubated as described previously. After 12–24 h of aerobic growth, oxygen transfer was limited by reducing the agitation speed to 100 rpm and fitting the shake flasks with sterile glass air traps.

Batch fermentations

Fermentations were carried out at 45°C and pH 5.0 in a 5-L Braun Biostat-B fermenter containing 4 L of MYFM. The fermenter was inoculated with 100 ml of the shake flask culture. The fermenter had automatic pH control using KOH (2 M) and H_2SO_4 (10%). Glucose solution was sterilised separately and added to the fermenter prior to inoculation giving a glucose concentration of 15–300 g L^{-1}. To determine the nitrogen uptake rate, ammonium sulphate was not added to the fermentation medium and NH_4OH was used as a base. The residual nitrogen concentration was determined using an Orion ammonia electrode.

During the aerobic phase, agitation was maintained at 300 rpm and the air flow rate was 4 L min^{-1}. After approximately 12 h, the air flow was switched off (for oxygen-limited conditions) or replaced with nitrogen (for anaerobic conditions) and the agitation was reduced to 150 rpm. Samples were taken during aerobic, oxygen-limited and anaerobic phases to determine the specific growth rate and specific ethanol production rate.

Continuous fermentation

The above procedure was followed, however during the oxygen-limited phase, fresh MYFM medium was added to give a dilution rate of 0.05–0.02 h^{-1}. A continuous fermentation with cell recycle was achieved using a 1-L sedimentation vessel in series after the fermenter vessel. A similar range of dilution rates (0.05–0.02 h^{-1}) was employed, based on a total working volume of 5 L (ie 4-L fermentation vessel plus 1-L sedimentation vessel). Sediment was recycled to the fermenter at a rate of 3 ml min^{-1}, the cell concentration in the recycle was dependent on the overall dilution rate and varied from 1–12 g L^{-1}.

Anaerobic growth

The ability of the strain to grow under anaerobic conditions was tested following the method of Visser *et al* [17]. Nominal 50-ml shake flasks containing 60 ml of MYFM medium plus 0.002% of the redox indicator resazurin were sealed with supaseal bungs and autoclaved at 120°C for 20 min. The redox indicator was decolorised. For supplemented flasks a solution of Tween 80 and ergosterol was dissolved in ethanol and injected into the shake flasks giving a resulting concentration of Tween 80 (0.66 g L^{-1}) and ergosterol (6 mg L^{-1}). Inoculum (2 ml) was aseptically injected into each flask. The syringe was left in place and indicated the production of carbon dioxide. The flasks were incubated at 45°C in a static incubator. Samples were taken by inverting the shake flask and extracting a 1-ml sample. The samples were assayed for optical density, glucose and ethanol. Due to the positive pressure in the flask it was unlikely that any oxygen diffused into the flask during this procedure and no evidence of colour change in the redox indicator was observed.

Analytical methods

Growth was monitored by measuring the absorbency at 660 nm of diluted broth samples and the biomass concentration determined from a standard curve. Glucose concentrations were found using commercial enzyme assays (Sigma). The ethanol concentration of clarified broth samples was analysed using a Perkin-Elmer capillary gas chromatograph (Foss Electric, Belfast, UK).

The oxygen uptake rate was determined by an oxygen mass balance over the fermenter. Oxygen analysis of the inlet and outlet air was carried out using a Rosemount Oxynos oxygen analyser (Williams Industrial Services, Belfast, UK). Gas flow rates were measured using a Cole-Palmer air flow meter for low air flow rates (0–1 L min^{-1}) and the integral Braun flow meter for higher air flow rates (2–10 L min^{-1}).

Results

The residual glucose concentration and ammonium hydroxide consumption rate during the aerobic phase of a batch fermentation were used to calculate the mean cell yield on glucose (0.2 g g^{-1}) and the mean cell yield on nitrogen (9.2 g g^{-1}) (Figure 1). The cell yield on nitrogen was comparable with figures given in the literature for yeast growth under nitrogen limitation eg 14 g g^{-1} for *Saccharomyces cerevisiae* and 12.5 g g^{-1} for *Candida utilis* [16]. The specific oxygen uptake rate, qOpX which was routinely measured over a number of fermentations varied between 0.3–0.6 g g^{-1} h^{-1} and resulted in a cell yield of between 0.0075 and 0.05 g g^{-1}. The cell yield on oxygen was dependent on the specific growth rate with high cell yields being observed

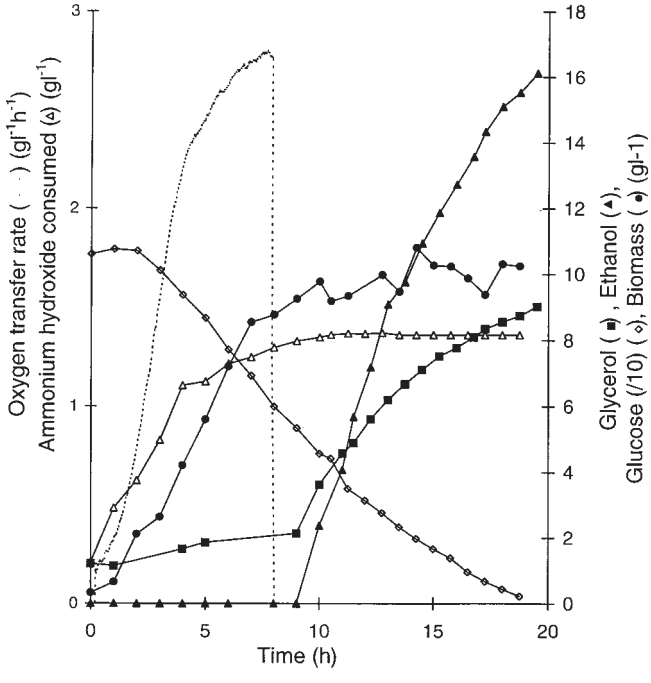

Figure 1 Batch fermentation of *K. marxianus* IMB3 at 45°C: ammonium hydroxide consumed (g L^{-1}) (–△–), residual glucose (g L^{-1}) (–◇–), biomass (g L^{-1}) (–●–), glycerol (g L^{-1}) (–■–) and ethanol (g L^{-1}) (–▲–). Air supply switched to nitrogen at 8 h, oxygen transfer rate (g L^{-1} h^{-1}) (----).

at high specific growth rates. The anaerobic phase was initiated after 8 h by replacing the air supply with nitrogen. Ethanol and glycerol were then produced until the glucose was depleted. During the anaerobic phase ammonium hydroxide consumption stopped and approximately 1 g L^{-1} of biomass was produced.

The strain was not capable of sustained growth under anaerobic conditions (Figure 2). The growth rate over the first 24 h was linear with a rate of 0.0114 g L^{-1} h^{-1} (regression value 0.995). During the following 48 h, the growth rate slowed reaching a final biomass concentration of 0.4 g L^{-1}. This represented a doubling of the initial biomass concentration. Ethanol was also produced, reaching a final ethanol concentration of 13 g L^{-1} (error ± 0.16 g L^{-1}). Addition of ergosterol and Tween 80, which are required for sustained anaerobic growth by *S. cerevisiae* [1,2], decreased the growth rate to 0.0068 g L^{-1} h^{-1} and led to a final biomass concentration of 0.33 g L^{-1}, however the final ethanol concentration reached was 14.6 g L^{-1} (error ± 0.4 g L^{-1}). The cell yield on glucose was 0.1 g g^{-1} in the unsupplemented flasks, a 50% decrease from the cell yield under aerobic conditions.

At glucose concentrations below 80 g L^{-1}, the specific growth rate followed Monod kinetics (regression value 0.9 following linearisation) with a maximum specific growth rate of 0.6 h^{-1} at 45°C and K_m of 0.065 g L^{-1}. Glucose concentrations above 80 g L^{-1} had an inhibitory effect on the specific growth rate. Specific ethanol productivity was slightly more tolerant to high osmotic activity with Michaelis–Menton kinetics observed at ethanol concentrations up to 100 g L^{-1} (Figure 3). The inhibition of the specific growth rate and the specific ethanol productivity at glucose concentrations of approximately 100 g L^{-1} is comparable with other strains of *K. marxianus* reported in the literature eg EMS-26 [3], NRRL 665 [7]. Specific ethanol

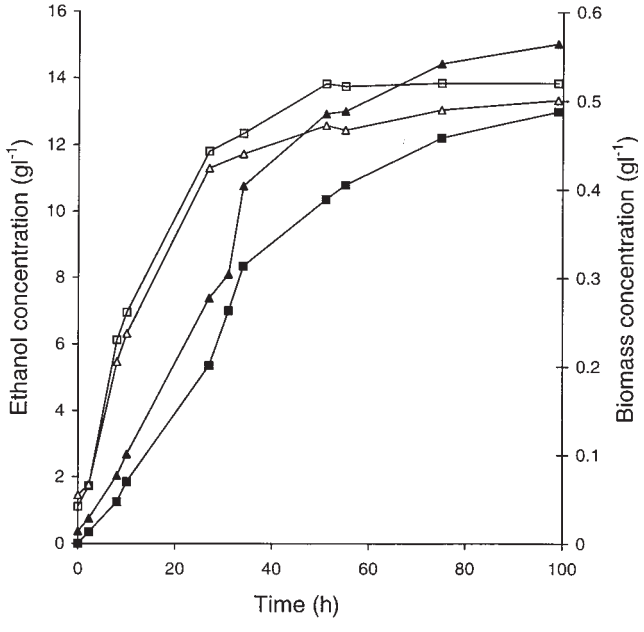

Figure 2 Batch growth of *K. marxianus* IMB3 at 45°C under anaerobic conditions: biomass (–△–) and ethanol (–▲–) in unsupplemented medium; biomass (–□–) and ethanol (–■–) in medium supplemented with ergosterol and Tween 80.

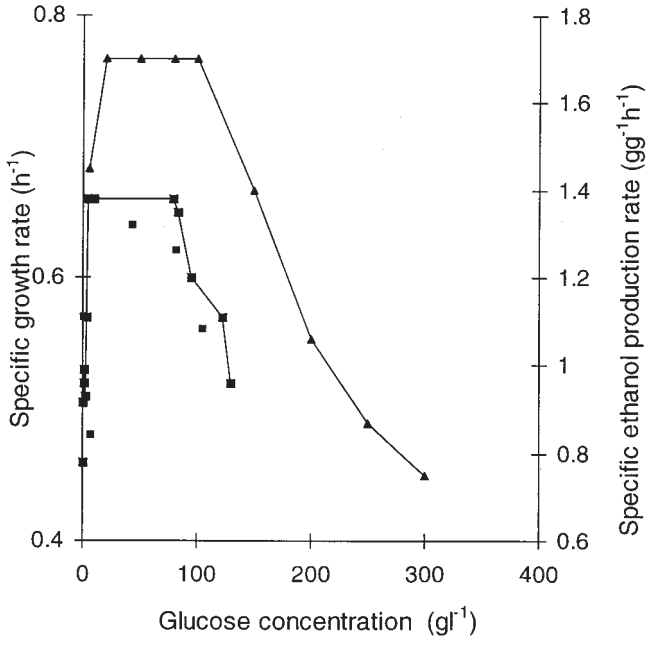

Figure 3 Effect of initial glucose concentration on maximum specific growth rate (–■–) in aerobic batch culture and maximum specific ethanol production rate (–▲–) in an oxygen-limited batch culture of *K. marxianus* IMB3 at 45°C.

productivity under aerobic conditions in the presence of excess sugar was very low (0.2 g g^{-1} h^{-1}) and only observed at high specific growth rates (0.4 h^{-1}).

The strain had a low ethanol tolerance reaching a maximum ethanol concentration of 40 g L^{-1} at 45°C. The specific ethanol production at low ethanol concentrations (<8 g L^{-1}) was 1.7 g g^{-1} h^{-1}. This decreased rapidly with ethanol concentration approximating to exponential decay (regression value 0.96) (Figure 4). The results show typical data from eight fermentations, error bars are displayed where the result is a mean reaction rate.

The minimum pH for growth was 2.5, although during one experiment cell death occurred at this pH. Cells remained viable at a pH of 2.0 for up to 2 h, thus the strain would be capable of withstanding the acid wash step used in industrial fermentation processes. For example, viability of *S. cerevisiae* was maintained after 2–4 h at pH values of 2–3 (personal communication HV Amorin, Fermentec s/c Itda, Brazil).

The low growth and ethanol production in continuous culture under oxygen-limited conditions is demonstrated in Figure 5. The maximum ethanol productivity from the system was 5.5 g h^{-1}. The specific ethanol productivity increased with dilution rate, resulting in an overall increase in ethanol productivity despite the decrease in biomass concentration (Figure 6). To increase the productivity of the system, the exit stream was fed to a sedimentation vessel and the settled biomass was recycled to the fermenter. This crude method of cell recycle was effective at low dilution rates; the cell concentration in the sediment was 12 g L^{-1}, which led to a doubling in the ethanol production rate. At higher dilution rates there was not sufficient residence time in the sedimentation vessel leading to minimal sedimentation rates. The biomass concentration in the sediment was

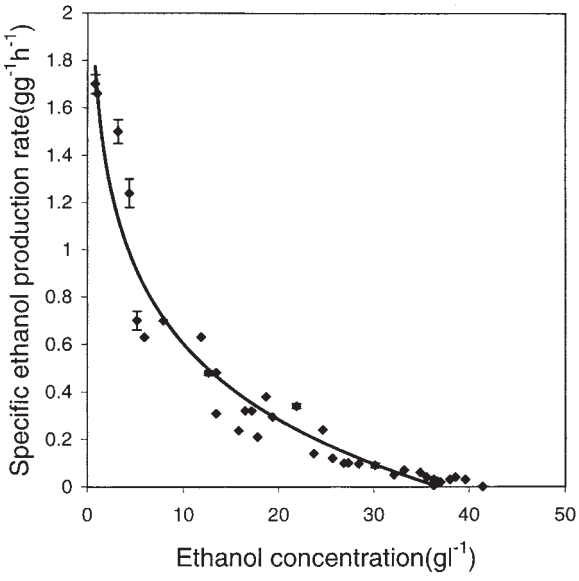

Figure 4 Correlation between endogenously produced ethanol and the recorded specific ethanol production rate during batch culture under oxygen-limited conditions at 45°C. The results were observed during eight fermentations, error bars are used when the result was the mean reaction rate measured at the given ethanol concentration. The glucose concentration was maintained in the range required for maximal specific ethanol production (20–100 g L^{-1}) (Figure 3).

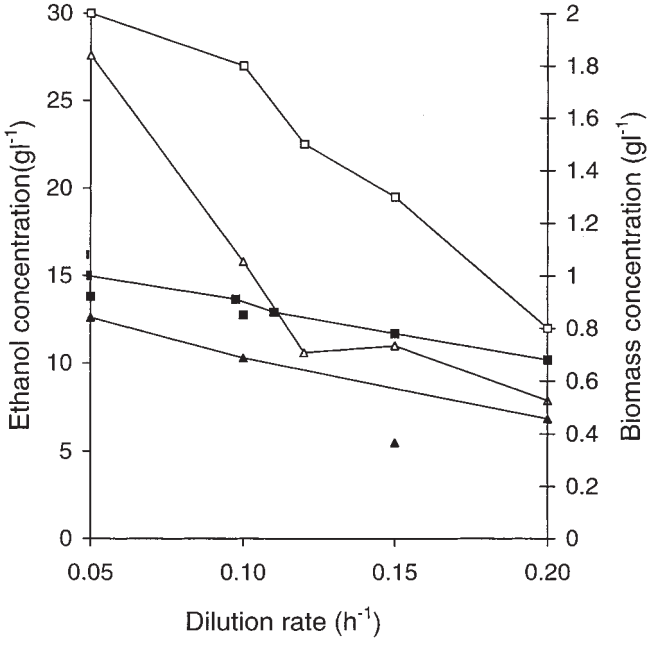

Figure 5 Continuous fermentation of *K. marxianus* IMB3 at 45°C biomass (–□–, –■–) and ethanol (–△–, –▲–) concentration without (closed symbol) and with biomass recycle (open symbol).

only 1 g L^{-1} thus ethanol productivity only increased slightly in comparison with the CSTR system. The increased productivity with dilution rate was therefore not observed in this system, resulting in a maximum ethanol productivity of 6.6 g h^{-1}. The specific ethanol productivity did increase with dilution rate as in the continuous system,

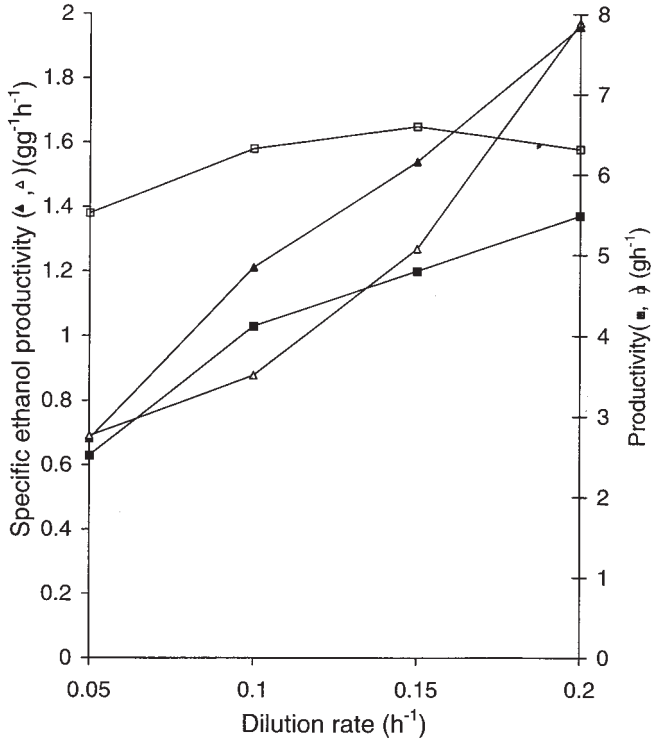

Figure 6 Effect of dilution rate on specific ethanol productivity of *K. marxianus* IMB3 at 45°C during continuous fermentation (closed symbol) and with biomass recycle (open symbol).

however, it was consistently lower in the system with recycle (Figure 6), this could be a reflection of the stress that the cells experienced in the sedimentation vessel with no pH or temperature control.

Discussion

Use of the thermotolerant yeast strain *K. marxianus* IMB3 has been proposed as a method of improving the efficiency of fuel ethanol production from biomass. It has been demonstrated that yields and tolerance to pH and osmotic activity are comparable to *S. cerevisiae*. The ethanol tolerance of strains of *K. marxianus* is low compared to *S. cerevisiae* [3,13], and the tolerance of the isolate IMB3 to ethanol is typical for the strain. The primary advantage of using *K. marxianus* IMB3 is, therefore, the ability to operate at elevated temperatures. Batch fermentation is not viable for an industrial process using *K. marxianus* IMB3. The initial substrate concentration would be limited by the osmotolerance of the strain and the low ethanol tolerance would result in low final ethanol concentrations and consequently increased downstream processing costs. A simple continuous process, advocated by Qureshi and Manderson [15], was also rejected due to the incapacity of the strain for sustained growth under anaerobic conditions. The maximum specific ethanol production rate of 1.7 g g^{-1} h^{-1} was only observed under anaerobic or oxygen-limited conditions [10,11], therefore growth and fermentation are not compatible in this strain. For a continuous process a method of retaining biomass in the fermenter was required. Recycling biomass resulted in a higher cell concentration and

improved productivity, however, for an industrial process a more efficient cell separation process or immobilisation would be required. This would have the advantage that the majority of the substrate entering the system would be utilised for ethanol production, further improving the economics of the process. As a consequence of increased ethanol production, *in situ* ethanol recovery would be required to alleviate ethanol tolerance problems, this would be facilitated by operating at 45°C.

Acknowledgements

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References

1 Andreasen AA and TJB Stier. 1953. Anaerobic nutrition of *Saccharomyces cerevisiae* I. Ergosterol requirement for growth in a defined medium. J Cell Comp Physiol 41: 26–35.
2 Andreasen AA and TJB Stier. 1954. Anaerobic nutrition of *Saccharomyces cerevisiae* II. Unsaturated fatty acid requirement for growth in a defined medium. J Cell Comp Physiol 43: 271–281.
3 Ballesteros I, JM Olivia, JC Carrasco and M Ballesteros. 1994. Effect of media supplementation on ethanol production by simultaneous saccharification and fermentation process. Appl Biochem Biotech 45/46: 283–294.
4 Banat IM, P Nigam and R Marchant. 1992. Isolation of a thermotolerant yeast growing at 52°C and producing ethanol at 45°C and 50°C. World J Biotechnol 8: 259–263.
5 Banat IM and R Marchant. 1995. Characterisation and potential industrial applications of five novel, thermotolerant, fermentative yeast strains. World J Microbiol Biotech 11: 304–306.
6 Barnett JA, RW Payne and D Yarrow. 1990. Yeasts: Characteristics and Identification, 2nd edn. Cambridge University Press, Cambridge.
7 Dale MC, A Eagger and MR Oakes. 1994. Immobilized *Kluyveromyces marxianus* in whey permeate concentrate. Proc Biochem 29: 535–544.
8 Dornin T. 1994. Ethanol fuel still not viable. Search 25: 2416.
9 Geller HS 1985. Ethanol fuel from sugar cane in Brazil. Ann Rev Energy 10: 135–164.
10 Hack CJ, R Marchant and WS McClean. 1994. The use of oxygen uptake rate to optimise air feed rate to a continuous ethanol fermentation. Prehrambeno-technol Biotechnol Rev 4: 187–190.
11 Hack FJ and R Marchant. 1995. The effect of oxygen transfer rate on continuous ethanol fermentation by *Kluyveromyces marxianus*. Prehrambeno-technol Biotechnol Rev 33: 151–154.
12 Moon B. 1994. Transport energy in Australia. Energy Policy 22: 331–344.
13 O'Leary VS, C Sutton, M Bencivengo, B Sulligan and VH Holsinger. 1977. Influence of lactose hydrolysis and solids concentration on alcohol production by yeast in acid whey ultrafiltrate. Biotech Bioeng 19: 1689–1702.
14 Pimentel D. 1991. Ethanol fuels: energy security, economics and the environment. J Agric Environ Ethics 4: 1–13.
15 Qureshi N and GJ Manderson. 1995. Bioconversion of renewable resources into ethanol: an economic evaluation of selected hydrolysis, fermentation and membrane technologies. Energy Sources 17: 241–265.
16 Rose AH. 1979. Economic Microbiology 4. Microbial Biomass (AH Rose, ed), Academic Press, London, UK.
17 Visser W, WA Scheffers, WH Batenburg-van der Vegte and JP van Dijken. 1990. Oxygen requirements of yeasts. Appl Environ Microbiol 56: 3785–3792.
18 Watson PJ. 1990. Malawi experience in fuel ethanol production and utilization. Int Sug J 92: 59–61.
19 Worthy W. 1984. New fuel ethanol plant starts up. Chem Eng News 62: 25.