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The Effect of Oxygen Transfer Rate on Continuous Ethanol Fermentation by *Kluyveromyces marxianus*

Utjecaj brzine prijenosa kisika na kontinuirano alkoholno vrenje s pomoću *Kluyveromyces marxianus*

Catherine J. Hack and R. Marchant*

Biotechnology Research Group, University of Ulster at Coleraine, Cromore Road, Co. Londonderry, Northern Ireland, BT52 1SA, UK.

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Summary

The effect of oxygen transfer rate on the growth rate, the specific ethanol productivity and the volumetric ethanol productivity of the thermotolerant yeast ***Kluyveromyces marxianus*** *IMB3 in a continuous ethanol fermentation was investigated. Under anaerobic conditions the strain grew poorly, with a maximum biomass concentration of 0.4 g* L^{-1}*. A small increase in oxygen transfer to the culture led to an increase in both the specific growth rate and the specific ethanol productivity. Further increases in the oxygen transfer rate to the culture caused a linear increase in the growth rate but a rapid decrease in the specific ethanol productivity. The specific ethanol productivity decreased to a constant minimum value of 0.25 (±0.05) g ethanol (g cells* $h)^{-1}$*. An initial maximum volumetric ethanol productivity, 0.14 g* L^{-1} h^{-1} *was achieved at an oxygen transfer rate to the culture broth of 3 mmol* L^{-1} h^{-1}*. The volumetric ethanol productivity increased again as the biomass concentration continued to rise although the specific ethanol productivity had levelled, reaching a maximum of 0.44 g* L^{-1} h^{-1}*. The biomass concentration in a non-aerated chemostat was estimated using the cell yield on oxygen and the oxygen transfer rate to the broth.*

Sažetak

Pri kontinuiranom alkoholnom vrenju ispitan je utjecaj brzine prijenosa kisika na rast termotolerantnog kvasca ***Kluyveromyces marxianus*** *i ukupni kapacitet proizvodnje etanola. Pod anaerobnim uvjetima prinos soja bio je slab, s maksimalnim udjelom biomase od 0,4 g/L. Neznatno povećanje prijenosa kisika u kulturu dovelo je do porasta specifične brzine rasta i specifične proizvodnje etanola. Daljnje povećanje brzine prijenosa kisika u kulturu uzrokovalo je linearni porast brzine rasta uz brzo smanjenje specifične proizvodnje etanola. Specifična proizvodnja etanola smanjila se do konstantne minimalne vrijednosti od 0,25 (±0,5) g etanola/g stanica na sat. Početna maksimalna proizvodnja etanola od 0,14 g* $(L\,h)^{-1}$ *postignuta je pri brzini prijenosa kisika u hranjivu podlogu od 3 mmol* $(L\,h)^{-1}$*. Volumetrijski se proizvodnja etanola ponovno povećala kada se kontinuirano povećavala koncentracija biomase, iako se specifična proizvodnja etanola ustalila dostigavši maksimum od 0,44 g* $(L\,h)^{-1}$*. Koncentracija biomase u neaeriranom kemostatu utvrđena je na osnovi prirasta stanica, ovisno o kisiku i brzini prijenosa kisika u hranjivu podlogu.*

Introduction

There has been a continued interest in using strains of *Kluyveromyces* for ethanol production due to their ability to grow and ferment at elevated temperatures. The recent isolation of thermotolerant fermentative yeast strains of *Kluyveromyces marxianus (1)* was believed to facilitate fermentation in warm climates and improve *in situ* ethanol recovery. The isolate designated *K. marxianus* IMB3 has been shown to grow and produce ethanol at 45 °C from sucrose *(2)*, lactose *(3)* and cellobiose *(4)*.

Kluyveromyces marxianus is a facultative fermentative yeast, i.e. it will produce ethanol under anaerobic conditions. Anaerobic oxidation of glucose is energetically inefficient, producing only 2 moles of ATP per mole of glucose compared to 38 moles of ATP under complete aerobic oxidation. Only *Saccharomyces cerevisae* has the capacity for rapid growth under anaerobic conditions, and then requires the addition of sterol and fatty acids to the media as these vital cell membrane components

* To whom correpondence should be addressed

can not be produced in the absence of oxygen. *Torulospora delbrueckii* and *Candida tropicalis* have been reported as capable of slow growth under anaerobic conditions (μ_{max} 0.03 h^{-1} and 0.05 h^{-1} respectively) *(5)*. Results for other strains are often contradictory, the major problems being the definition and the maintenance of strictly anaerobic conditions, and differences in inoculum preparation. A method for ensuring anaerobiosis has been reported *(5)* in which resazurin was used as a redox indicator.

This paper investigates the effect of the rate of oxygen transfer to the culture medium on growth, specific and volumetric ethanol productivity of *Kluyveromyces marxianus* IMB3 and discusses the implications for continuous ethanol production in a chemostat.

Materials and Methods

Microorganism and maintenance

Strains of *Kluyveromyces marxianus* var. *marxianus* were obtained from soil samples collected from ground at Associated Distilleries, Northern India. They were selected by enrichment culture and maintained on nutrient agar slopes at 4 °C *(1)*.

Media and inoculum preparation

Inocula were prepared in 100 mL shake flasks containing 50 mL of yeast fermentation media (MYFM): yeast extract (3 g L^{-1}); peptone (3 g L^{-1}); KH_2PO_4 (4 g L^{-1}); $(NH_4)_2SO_4$ (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}. Flasks were inoculated with a single colony of *K. marxianus* from agar slopes and incubated for 12–24 hours at 45 °C and 200 rpm in a New Brunswick incubated orbital shaker. The flasks were used to inoculate a Braun Biostat-B chemostat (5 L) or further shake flasks.

Anaerobic growth

Nominal 50 mL Erlenmeyer flasks were almost filled with 60 mL MYFM media containing 0.002 % of the redox indicator resazurin and 80 g L^{-1} glucose. Resazurin is strongly coloured (orange or purple depending on the pH of the medium) at low redox potentials (E_0 = –42 mV). The flasks were sealed with 37 mm SUBA-SEAL bungs (William Freeman Ltd. patent no. 470720) and autoclaved for 20 minutes at 120 °C. Some flasks decolorised, some remained coloured and some flasks exploded. Flasks which had not decolorised during sterilisation were vigorously sparged with oxygen free nitrogen, however, no decolorisation occurred and the flasks were discarded. Ergosterol and Tween 80 were dissolved in ethanol and added to each flask to give a resulting concentration of Flask A: 0,0; Flask B: 3,330 mg L^{-1}; Flask C: 6,660 mg L^{-1} and Flask D: 12,1320 mg L^{-1}, respectively. A 2 mL aliquot of inoculum was injected into each flask via a 5 mL syringe. The syringe was left in place to allow for sampling and to indicate the production of CO_2. The flasks were incubated at 45 °C in a static incubator, the experiment was repeated using an incubator-shaker at 50 rpm with no significant difference in the results (results not shown). Samples were removed at intervals by gently agitating the flask and inverting. It was thought unlikely that air would enter the flask during sampling due to the slight positive pressure caused by CO_2 production. Samples were analysed for biomass, ethanol and glucose concentrations. The results shown are the mean taken from replica flasks. Error bars are displayed where significant.

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 (150 g L^{-1} glucose). The fermenter was inoculated with 50 mL of the shake flask culture. The pH and temperature were monitored and controlled using Braun software. For fermentations at fixed oxygen transfer rates the air flow rate to the vessel (0–1 L min^{-1}) was controlled and monitored using Cole-Palmer flow meters and the agitation was set at 150 rpm. Biomass and ethanol concentrations were measured periodically to determine the specific ethanol productivity.

Continuous Fermentation

The above procedure was repeated with an air flow rate of 4 L min^{-1} and an agitation rate of 250 rpm. After 24 hours of aerobic growth the fermentation was switched to continuous operation. Two 20 L carboys containing glucose and MYFM media were connected aseptically to the fermenter. The solutions were pumped to the fermenter via Watson-Marlow pumps, giving a glucose concentration in the fermenter of 100 g L^{-1} and a dilution rate of 0.1 h^{-1}. After 24 hours of continuous aerobic operation the ethanol and cell concentration were measured every 2 hours until steady-state values were reached. The air was then switched off and the agitation reduced to 150 rpm. The dilution rate was controlled at 0.05–0.175 h^{-1} and the steady-state ethanol and biomass concentrations were recorded.

Determination of oxygen transfer rate

The oxygen transfer rate to the vessel was measured during aeration via sparging with air and during passive oxygen transfer across the surface of the broth. In both sets of experiments the dissolved oxygen concentration was measured using an Ingold polarographic oxygen electrode. The air flow rate to the vessel was monitored and controlled using Cole-Palmer gas flow meters and maintained at 0–1 L min^{-1} and the exit gas oxygen concentration measured using a Rosemount Oxynos oxygen monitor. The agitation rate was set at 150 rpm. During passive oxygen transfer the air was switched off and the broth sparged with nitrogen until a zero reading registered on the pO_2 probe. The nitrogen was then switched off and the change in dissolved oxygen monitored with time at stirrer speeds of 50–200 rpm (Fig. 1).

Analytical methods

The biomass concentration was determined from absorbance at 660 nm of diluted broth samples using a standard curve. The glucose concentration was checked

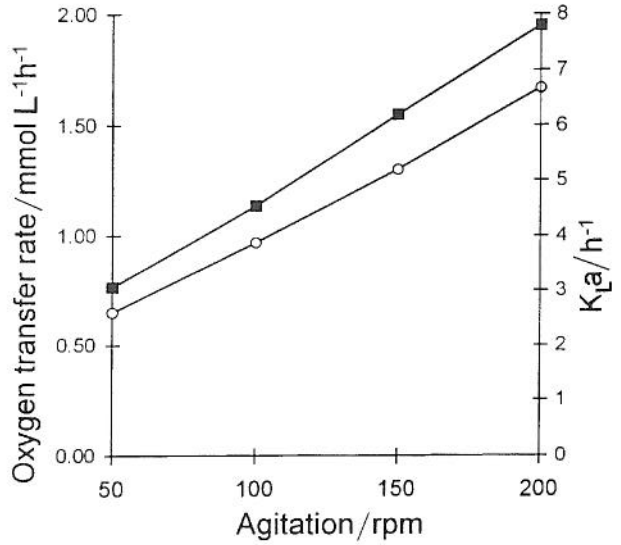

Fig. 1. Determination of oxygen transfer rate, mmol $L^{-1}h^{-1}$ (O) and K_La h^{-1} (■) for non-aerated fermenter
Slika 1. Određivanje brzine prijenosa kisika, mmol $L^{-1}h^{-1}$ (O) i K_La h^{-1} (■) za nelinearni fermentor

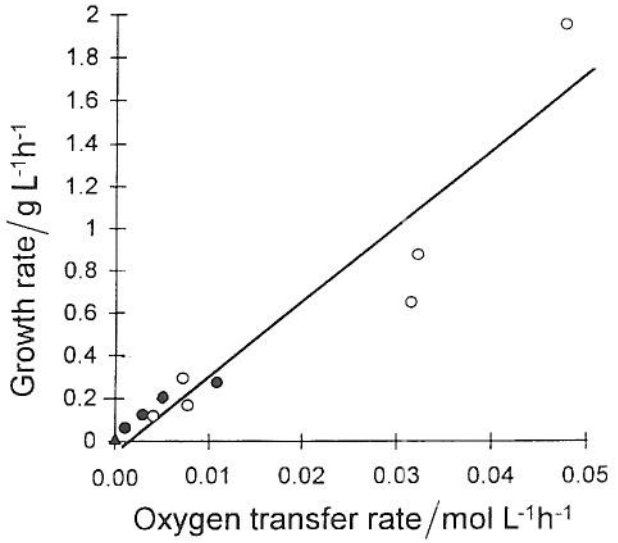

Fig. 2. Effect of oxygen transfer rate on growth rate of *K. marxianus*: anaerobic growth (▲), sparged air (O), surface oxygen transfer (●)
Slika 2. Utjecaj brzine prijenosa kisika na rast *K. marxianus*: anaerobni rast (▲), ubacivani zrak (O), prijenos kisika s površine (●)

in the continuous culture experiments using Dextrostix (Sigma). The ethanol concentration of clarified broth samples was analyzed using a Perkin-Elmer capillary gas chromatograph.

Results and Discussion

The maximum specific growth rate of the used strain on glucose in defined media under strict anaerobic conditions at 45 °C was 0.056 h^{-1} and the specific ethanol productivity was 0.7 g ethanol/g cells · h. Addition of ergosterol and Tween 80 decreased the specific growth rate but led to a higher final ethanol concentration. These results compare poorly with a maximum specific growth rate under aerobic conditions of 0.6 h^{-1} *(1)* and a specific ethanol productivity under non-aerated conditions following aerobic growth of 1.7 g ethanol/g cells · h (unpublished results).

Growth and ethanol production were investigated at intermediate oxygen transfer rates, whilst maintaining the dissolved oxygen concentration in the broth at zero. Growth rate increased linearly with oxygen transfer rate (regression value 0.9) (Fig. 2) giving a cell yield on oxygen of 36 g (mol O_2) ^{1}.

The theoretical yield of yeast on oxygen can be determined from the stoichiometry of the oxidation process. Assuming a cell molecular weight of 24.6 and a cell yield on glucose of 0.5 g (g glucose)$^{-1}$, the cell yield on oxygen is 42 g (mol O_2)$^{-1}$ *(6)*.

The specific ethanol productivity had an optimum of 1.5 g ethanol (g cells h) ^{1} at an oxygen transfer rate of 1 mmol $L^{-1}h^{-1}$ (Fig. 3), it then decreased rapidly with oxygen transfer rate, reaching a minimum of 0.26 g (g cells)$^{-1}$ h^{-1} at an oxygen transfer rate of 20 mmol $L^{-1}h^{-1}$. Further increases in the oxygen transfer rate (up to 50 mmol) did not affect the specific ethanol productivity. The method of oxygen transfer either by sparging or through passive oxygen transfer across the surface of the broth did not affect the growth or the specific ethanol productivity.

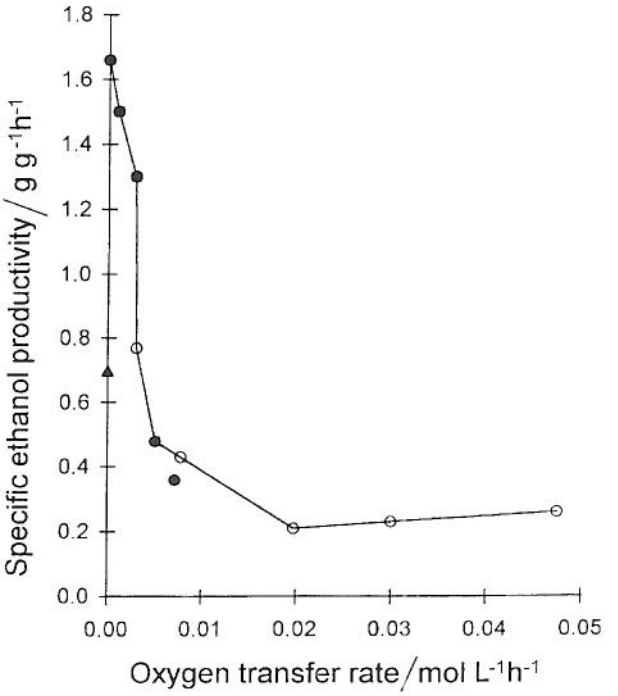

Fig. 3. Effect of oxygen transfer rate on specific ethanol productivity of *K. marxianus*: anaerobic growth (▲), sparged air (O), surface oxygen transfer (●).
Slika 3. Utjecaj brzine prijenosa kisika na specifičnu proizvodnju etanola u *K. marxianus*: anaerobni rast (▲), ubacivani zrak (O), prijenos kisika s površine (●).

The volumetric ethanol productivity has an initial maximum of 0.14 g L^{-1} h^{-1} at an oxygen transfer rate of 3 mmol L^{-1} h^{-1}. Under these conditions the high specific ethanol productivity compensates for low biomass con-

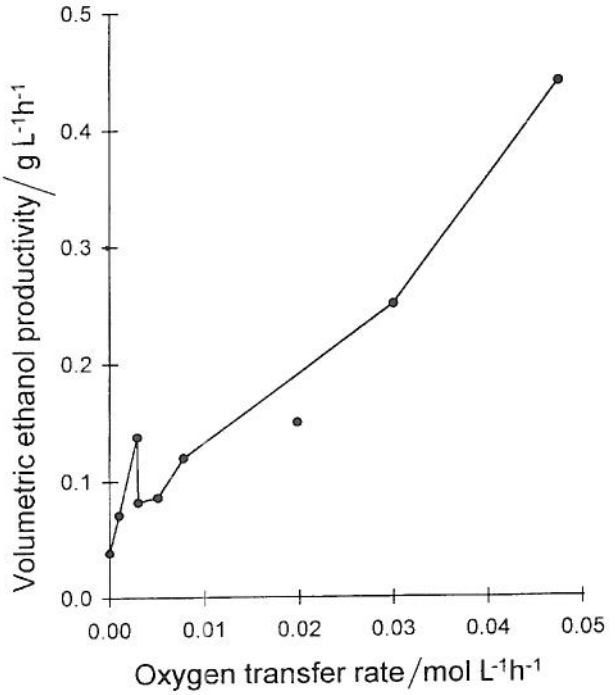

Fig. 4. Effect of oxygen transfer rate on volumetric ethanol productivity of *K. marxianus*
Slika 3. Utjecaj brzine prijenosa kisika na proizvodnju etanola u *K. marxianus*

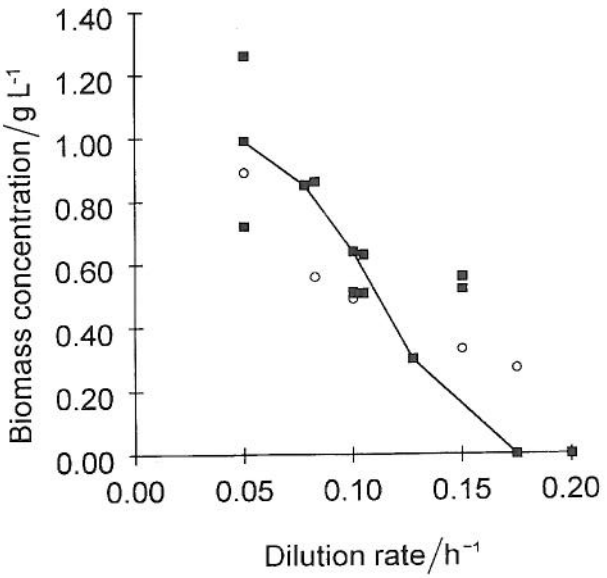

Fig. 5. Biomass concentration of *K. marxianus* in continuous fermentation: experimental results (■), estimated from experimental yield on oxygen (O)
Slika 5. Koncentracija biomase *K. marxianus* pri kontinuiranom vrenju: eksperimentalni podaci (■), promijenjene vrijednosti na osnovi experimentalnih iskorištenja kisika (O)

centration. As the biomass concentration continues to increase linearly with oxygen transfer rate and the specific ethanol productivity decreases to a constant level, a second increase in volumetric productivity is observed (Fig. 4). The size of this maximum will be determined by the biomass concentration and will ultimately be limited by the oxygen transfer characteristics of the fermentation vessel. The maximum productivity recorded in the Braun chemostat under full aerobic conditions was 6.2 g $L^{-1}h^{-1}$.

The biomass concentration in a non–aerated chemostat at dilution rates of 0.05–0.175 h^{-1} are given in Fig. 5. The maximum dilution rate in this system was 0.15 h^{-1}. Fig. 5 also shows the biomass concentration predicted from the rate of oxygen transfer to the broth, assuming the cell yield on oxygen was 36 g mol^{-1}. The total amount of oxygen available to the cells was the sum of the oxygen transferred across the surface of the broth plus the oxygen concentration in the feed entering the chemostat.

Conclusions

Growth and ethanol production were not compatible in the strain. Poor growth under strictly anaerobic conditions indicated that growth in the non-aerated ethanol production stage of a continuous fermentation was maintained by the oxygen transfer rate across the surface of the media plus the oxygen in the feed. This is confirmed by the estimation of the cell concentration in the chemostat from the cell yield on oxygen. Maximum productivity could be achieved in an aerated vessel. This maximum would be determined by the cell concentration and thus the oxygen transfer rate to the vessel. This system would be operating at low specific activities and low ethanol yields.

For an efficient continuous ethanol process alternative methods would have to be employed to maintain a high biomass concentration in the reactor. Process configurations explored by the group are the use of two fermenters in series, cell recycle *(7,8)* and immobilisation *(9)*.

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