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ORIGINAL PAPER

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Fed-batch fermentation for production of *Kluyveromyces marxianus*FII 510700 cultivated on a lactose-based medium

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Abstract A strain of *Kluyveromyces marxianus* was grown in batch culture in lactose-based media at varying initial lactose concentrations (10–60 g L^{-1}) at 30°C, pH 5.0, dissolved oxygen concentrations greater than 20%. Increasing the concentration of mineral salts three-fold at 40 g L^{-1} and 60 g L^{-1} initial lactose concentration showed only a small increase in the yield of biomass, from 0.38 g g^{-1} to 0.41 g g^{-1}, indicating that the initial batch cultures were not significantly nutrient-(mineral salts)-limited. A relatively high biomass concentration (105 g L^{-1}) was obtained in fed-batch culture following extended lactose feeding. An average specific growth rate (0.27 h^{-1}), biomass yield (0.38 g g^{-1}) and overall productivity (2.9 g L^{-1} h^{-1}) were obtained for these fed-batch conditions. This fed-batch protocol provides a strategy for achieving relatively high concentrations and productivities of *K. marxianus* on other lactose-based substrate streams (e.g., whey) from the dairy industry.

Keywords Fed-batch culture · *Kluyveromyces marxianus* · Lactose-based media · Whey

Introduction

Among the various options for whey treatment/utilization, the cultivation of *Kluyveromyces* spp offers the potential for both lowering its chemical oxygen demand (COD) and converting constituent lactose into microbial biomass which may have commercial value as a source of yeast autolysates, protein and other bio-products [22].

Strains of *Kluyveromyces* or their synonyms, *K. fragilis* and *Saccharomyces fragilis*, have been considered the most suitable for bio-conversion of lactose in whey [2, 4, 20]. However, incomplete or slow fermentations have been observed for many *Kluyveromyces* strains, when concentrated whey or lactose-enriched substrates have been employed [12, 32]. These effects have been attributed variously to the toxicity of the ethanol produced and/or to inhibition by high salt concentrations, resulting in elevated osmotic pressure [12]. Furthermore, it has been reported that increases in lactose concentrations can lead to the accumulation of pyruvate resulting from the greater glycolytic flux in these yeasts, thereby causing a reduction in final biomass yields [3].

Fed-batch culture offers the potential to maintain growth conditions while maintaining relatively low substrate (lactose) concentrations and associated salts and by-products early in the fermentation. Such fed-batch cultures involve sequential addition of nutrients at a rate maintaining low substrate concentrations and have been reported for the production of yeast biomass from *Saccharomyces uvarum*, the production of extracellular inulinase from *K. marxianus* and the production of ethanol and biomass from *K. fragilis* [9, 14, 23, 29]. As shown by Siso [28] and by Belem and Lee [3], relatively high biomass concentrations and yields can be achieved in fed-batch culture by preventing the imbalance of glycolytic metabolism over oxidative metabolism. In addition, concentrated medium can be added at a rate that maintains a low substrate (lactose) concentration and minimizes inhibition effects. Since most of the information available on *Kluyveromyces* spp is limited to batch-culture studies [21, 30], the objective of this investigation is to extend batch-culture studies to fed-batch culture in order to maximize biomass concentrations of *K. marxianus* which may be used subsequently for higher-value products. Recent studies by our group have established the potential of *K. marxianus* biomass as an alternative source to *S. cerevisiae* for yeast

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autolysates, alkali-insoluble glucans and a natural bio-emulsifier [17–19]. Furthermore, high-productivity fed-batch culture could also be an effective strategy for whey-waste pollution control.

Materials and methods

Yeast strain and maintenance

A strain of *K. marxianus* FII 510700 (FRR 1586) previously cultivated on casein whey [15] was obtained from the Culture Collection of the University of New South Wales (UNSW 248; World Data Centre for Microorganisms). The yeast was cultured and maintained as reported in our earlier studies [17].

Inoculation and preparation

Seed cultures were prepared by inoculating a colony from a solid culture into 50-mL flasks containing 10 mL sterile lactose-based medium (described below). The culture was incubated in an orbital shaking incubator (New Brunswick, USA) rotating at 180 rpm and maintained at 30°C for 14 h. After incubation, the culture broth was added to 500-mL flasks containing 90 mL sterile medium and incubated in the orbital incubator (180 rpm) at 30°C for 24 h.

Medium composition

The medium was deproteinated lactose powder (DPP; Murray Goulburn Co-operative, Melbourne, Australia) containing (per litre): 10–60 g lactose, 5 g $(NH_4)_2SO_4$, 2 g $MgSO_4$ $7H_2O$, 4 g KH_2PO_4, 2 g yeast extract.

Batch and fed-batch cultures

A 5-L Biostat B fermenter (B. Braun Biotech International, Melsungen) was used for all experiments. The fermenter was equipped with controllers for pH, temperature, agitation and dissolved oxygen (DO) concentration. Batch cultures were carried out by adding a 3% inoculum to 3 L sterile medium in a pre-sterilized Biostat B fermenter (121°C, 15 min). The temperature and pH were controlled at 30°C and 5.0, respectively, while the agitation speed automatically varied at a fixed air flow rate during the culture to maintain the DO concentration above 20% air saturation.

The fed-batch culture was started as a batch culture following the addition of a 3% inoculum into medium containing 10 g L^{-1} initial lactose concentration. When the lactose concentration was reduced to approximately 0.5 g L^{-1} by growth of the yeast, concentrated medium was fed, containing (per litre): 360 g lactose, 72 g $KH_2PO_4{\cdot}7H_2O$, 36 g $MgSO_4$, 90 g $(NH_4)_2SO_4$, 36 g yeast extract. This concentrated medium was fed stepwise at intervals of 1–2 h with increases of approximately 20 g L^{-1} lactose concentration. Samples taken just prior to lactose addition confirmed that the residual lactose concentration in the vessel was close to zero at these times. At cell concentrations above 40 g L^{-1}, pure oxygen was mixed with air to meet the high COD during fed-batch operation.

Estimation of biomass concentration

Biomass concentration was estimated by pipetting 4-mL samples into 7-mL pre-weighed dry tubes and centrifuging at 5,000 *g* for 5 min. Following removal of the supernatant, cells were washed (twice) by resuspending in milli-Q water and centrifuging as previously described. The washed cells were dried in an oven at 105°C for 48 h, after which the tubes were re-weighed and the cell dry weights (DCW) estimated.

Determination of concentrations of sugars and ethanol

The concentrations of lactose, galactose, glucose and ethanol in the culture broth were determined by high-performance liquid chromatography (HPLC). The instrument used was a Waters HPLC system consisting of a Waters 510 pump, a Waters 486 tunable detector, a Waters 715 Ultra Wisp sample processor and an automation interface with a computer using Maxima 820 software.

A Biorad HPX-87P column (250 mm) was used for HPLC analysis. The column was eluted at 85°C with degassed double-deionized water at a flow rate of 0.6 mL min^{-1}. Standards of known concentrations of lactose, galactose, glucose and ethanol (Sigma Chemicals) dissolved in milli-Q water were used. Samples were filtered through a 0.45-μm membrane prior to injection into the column. The concentration of ethanol was confirmed by gas chromatography (chapter 26 in [1]). The instrument was a Packard model 427 fitted with a glass column (1.2 m long, 6 mm ID, packed with Porapak Q 100/120 mesh; Alltech) and was run isothermally at 180°C. Ethanol was carried by nitrogen gas at 1 kPa and detection was by a flame ionization detector (FID) at 220°C. The injection port was also set at 220°C.

Determination of concentrations of organic acids

The concentrations of organic acids in the culture broth were determined by using the Waters HPLC previously described. A Biorad HPX-87H column was used for the determination of organic acid concentrations. The column was eluted at 50°C with dilute degassed H_2SO_4 at a flow rate of 0.5 mL min^{-1}. Standards (0.1–0.7 g L^{-1}) of acetic, citric, lactic, fumaric, malic, oxalic, propionic,

Table 1 Effect of increasing initial lactose concentration in DPP medium on the growth parameters of *K. marxianus* FII 5107000. The yeast was grown in batch culture at pH 5.0, 30°C, DO >20%. The biomass yield was estimated based on lactose consumed

Kinetic parameter	Lactose concentration			
	10 L^{-1}	20 L^{-1}	40 L^{-1}	60 L^{-1}
Average specific growth rate, μ_m (h^{-1})	0.37	0.35	0.37	0.36
Average specific lactose uptake rate, q_s (g g^{-1} h^{-1})	0.40	0.35	0.37	0.36
Biomass yield, $Y_{x/s}$(g g^{-1})	0.47	0.45	0.37	0.38
Biomass concentration (g L^{-1})	4.70	9.00	15.0	23.0
Biomass productivity (g L^{-1} h^{-1})	0.47	0.75	1.26	1.43
Maximum ethanol concentration (g L^{-1})	–	–	3.20	3.48

pyruvic, succinic and tartaric acid (Sigma Chemicals) were filtered through a 0.45-μm filter prior to injection into the column.

Evaluation of kinetic parameters

Kinetic parameters for the growth, biomass production and lactose uptake of *K. marxianus* in batch and fed-batch culture were calculated using standard methods [26].

Results

Batch culture growth: effect of increasing lactose concentration

A summary of the kinetic data for the production of biomass and the uptake of lactose over the initial concentration range 10–60 g L^{-1} at 30°C, pH 5.0 and DO >20% air saturation are presented in Table 1. Typical data are shown in Fig. 1 for medium containing 40 g L^{-1} lactose. Relatively high biomass yields were obtained in the concentration range 10–20 g L^{-1} lactose in DPP medium. Increasing the lactose concentration from 40 g L^{-1} to 60 g L^{-1} resulted in some decrease in biomass yield, together with the production of low amounts (3.20–3.48 g L^{-1}) of ethanol. Glucose and galactose were not detected. Low levels of organic acids (0.1–0.3 g L^{-1}) were detected in these fermentations. The fermentations were complete within 12–16 h.

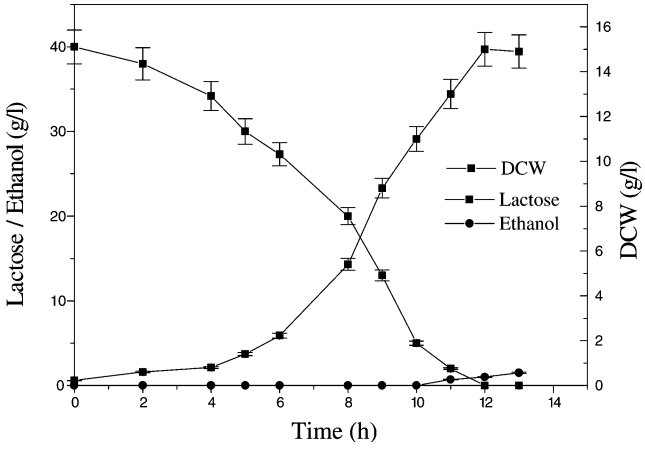

Fig. 1 Batch culture of *K. marxianus* at 40 g L^{-1} initial lactose concentration. Data points are means of duplicate analyses

Effect of increasing concentrations of other nutrients

The effect of increasing the concentration of mineral salts in DPP medium on the production of *K. marxianus* biomass in batch culture is illustrated in Table 2 for medium containing 40 g L^{-1} and 60 g L^{-1} lactose. The results indicate that the yield of *K. marxianus* biomass was only increased by 10% after tripling the nutrient concentrations [from, (per litre): 4 g KH_2PO_4, 2 g $MgSO_4{\cdot}7H_2O$, 5 g $(NH_4)_2SO_4$, 2 g yeast extract; to (per litre) 12 g KH_2PO_4, 6 g $MgSO_4{\cdot}7H_2O$, 15 g $(NH_4)_2SO_4$], while maintaining the yeast extract at 2 g L^{-1}. A much smaller amount of ethanol (<0.3 g L^{-1}) was produced with the increased mineral salts concentration, indicating that some fermentation may have resulted previously from a growth limitation due to nutrient depletion.

Fed-batch culture

K. marxianus was cultivated in fed-batch culture to maximize the production of biomass. Concentrated nutrients were fed stepwise to maximize biomass productivity and minimize the production of ethanol. Fed-batch culture was initiated following batch culture at an initial lactose concentration of 40 g L^{-1}. A maximum concentration of 105 g L^{-1} biomass in 35 h (Figs. 2, 3) was achieved using fed-batch culture. A summary of results of fed-batch culture in comparison with batch culture is presented in Table 3.

Discussion

Deproteinized whey has been used extensively for the production of enzymes [13], autolysed yeast extract [22] and lactic acid [27]. In such batch culture fermentations, the average specific growth rate for *K. marxianus* was reported to be 0.43 h^{-1}. This compares to the value obtained in our batch culture of 0.36 h^{-1}.

Table 2 Effect of increasing mineral salts concentration on the kinetics of *K. marxianus* FII 510700 biomass. The yeast was grown in batch culture at pH 5.0, 30°C and 20% DO

Kinetic parameter	Lactose	
	40 g L^{-1}	60 g L^{-1}
Average specific growth rate, μ_m (h^{-1})	0.35	0.35
Biomass yield, $Y_{x/s}$ (g g^{-1})	0.41	0.41
Biomass concentration (g L^{-1})	16.4	24.6
Biomass productivity (g L^{-1} h^{-1})	1.26	1.54
Maximum ethanol concentration (g L^{-1})	< 0.30	< 0.30

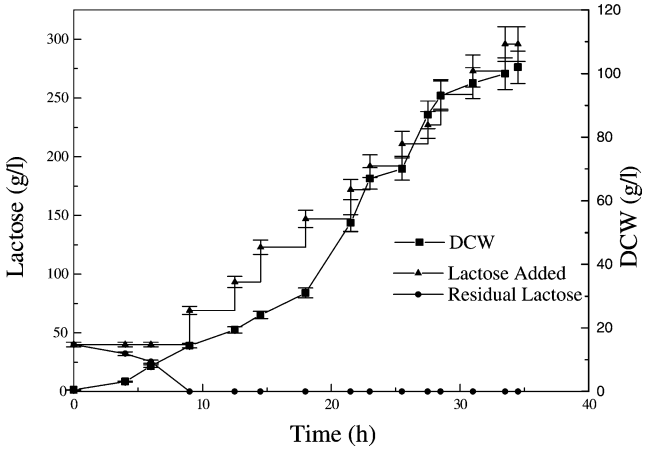

Fig. 2 Fed-batch cultivation of *K. marxianus* in DPP medium. Data points are means of duplicate analyses

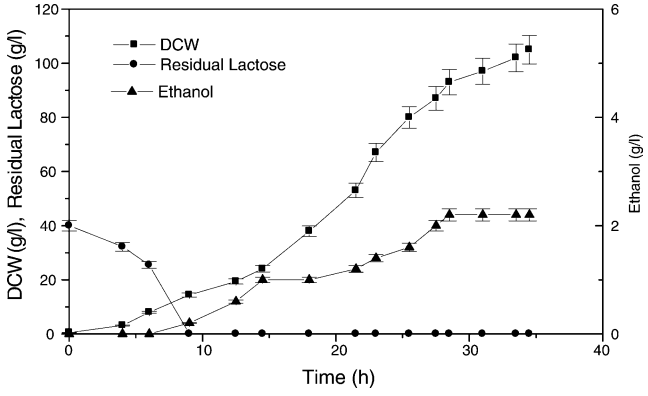

Fig. 3 DCW vs time during fed-batch fermentation of *K marxianus* (30°C, pH 5.0)

Biomass yields of batch cultures in DPP medium were 0.47 g g^{-1} at 10 g L^{-1} initial lactose concentration, 0.45 g g^{-1} at 20 g L^{-1} initial lactose concentration and 0.37 g g^{-1} at 40 g L^{-1} initial lactose concentration. These results are much higher than those obtained by Giec and Kosikowski [11] and Duvnjak et al. [7] but are similar to those of Orban et al. [24] and Belem and Lee [3]. Even under fully aerobic conditions (> 20% DO concentration), ethanol was detected at initial lactose concentrations of 40 g L^{-1} and 60 g L^{-1}. Tripling the nutrient concentrations showed only a small increase in the yield of *K. marxianus* biomass, with a maximum yield of 0.41 g g^{-1} being achieved, compared to 0.37–0.38 g g^{-1} in previous batch culture studies. However, this observation could vary in different strains of *K. marxianus*.

Low levels of organic acids, viz. 0.03 g L^{-1} pyruvic acid, 0.01 g L^{-1} malic and acetic acid, together with small amounts (< 0.01 g L^{-1}) of citric, acetic, propionic and fumaric acid were detected in batch-culture broths of *K. marxianus*. Production of organic acids is, however, a normal event during the growth of yeasts [31].

It has been long established that high sugar concentrations in batch cultured yeasts can result in Crabtree repression, which inhibits respiratory enzymes and increases ethanol production [5, 10]. This can be overcome by fed-batch cultivation, where essential nutrients can be fed incrementally to the bioreactor during cultivation [16, 25]. In this study, fed-batch culture was used to increase biomass production from 15 g L^{-1} DCW in batch culture to a maximum of 105 g L^{-1} DCW and to minimize any effects of Crabtree repression or substrate (lactose) inhibition, which resulted in the production of low levels of ethanol (Table 1) and a reduced growth rate in the batch culture.

The biomass production of 105 g L^{-1} DCW in fed-batch culture produced in 35 h represented a yield of 0.38 g g^{-1} and a productivity of 2.9 g L^{-1} h^{-1}. These results are higher than those reported by Belem and Lee [3] and Nor et al. [23], who obtained maximum production of

Table 3 Summary of results of batch culture in comparison to fed-batch culture of *K. marxianus* FII 510700 using DPP medium. The batch culture was grown at 40 g L^{-1} initial lactose concentration, 30°C, pH 5.0, DO > 20%

Kinetic parameter	Method of culture		
	Batch	Fed-batch 1	Fed-batch 2
Avereage specific growth rate, μ_m (h^{-1})	0.37	0.27	0.27
Average specific lactose uptake rate, q_s (g g^{-1} g^{-1})	0.66	0.92	0.95
Biomass concentration (g L^{-1})	15.0	70	105
Time (h)	12	29	34.5
Biomass yield, $Y_{x/s}$ (g g^{-1})	0.37	0.37	0.38
Biomass productivity (g L^{-1} h^{-1})	1.26	2.41	2.90

28 g L^{-1} and 69 g L^{-1} DCW and productivities of 2.42 g L^{-1} h^{-1} and 4.21 g L^{-1} h^{-1}, respectively, in similar studies with *K. fragilis*. The results however compare with those reported by Lee and Kim [16] for *Candida utilis* cultivated on molasses, where a biomass yield of 0.48 g g−1 and biomass productivity of 1.74 g L^{-1} h^{-1} were obtained. In comparison, only a maximum of 59 g L^{-1} biomass was produced from fed-batch fermentations of *S. cerevisiae* using hydrolysed waste cassava as the carbohydrate source [8], while repeated fed-batch fermentations of mixed yeast cultures of *Torula cremoris* and *C. utilis* were required to increase the overall biomass yield on lactose to 0.76 g g^{-1} over 60 h [6].

We therefore conclude that the fed-batch culture strategies reported in this paper offer a significant incentive for large-scale production of *K. marxianus* cultivated on lactose-based media at reduced costs. Furthermore, such fed-batch cultivations could be used as a treatment option for whey in a lactose-rich waste stream of the dairy industry.

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