Physiology of the yeast *Kluyveromyces marxianus* during batch and chemostat cultures with glucose as the sole carbon source

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Abstract

Growth, substrate consumption, metabolite formation, biomass composition and respiratory parameters of *Kluyveromyces marxianus* ATCC 26548 were determined during aerobic batch and chemostat cultivations, using mineral medium with glucose as the sole carbon source, at 30 °C and pH 5.0. Carbon balances closed within 95–101% in all experiments. A maximum specific growth rate of 0.56 h^{-1}, a biomass yield on glucose of 0.51 g g^{-1}, and a maximum specific consumption of oxygen of 11.1 mmol g^{-1} h^{-1} were obtained during batch cultures. The concentration of excreted metabolites was very low at the culture conditions applied, representing 6% of the consumed carbon at most. Acetate and pyruvate were excreted to a larger extent than ethanol under the batch conditions, and the protein content accounted for 54.6% of the biomass dry weight. Steady states were obtained during chemostats at dilution rates of 0.1, 0.25 and 0.5 h^{-1}. At the two former dilution rates, cells grew at carbon limitation and the biomass yield on glucose was similar to that obtained under the batch conditions. Metabolite formation was rather low, accounting for a total of 0.005 C-mol C-mol^{-1} substrate. At 0.5 h^{-1}, although the biomass yield on glucose was similar to the value obtained under the above-mentioned conditions, the cultivation was not under carbon limitation. Under this condition, 2-oxoglutarate, acetate, pyruvate and ethanol were the prevalent metabolites excreted. Total metabolite formation only accounted to 0.056 C-mol C-mol^{-1} of substrate. A very high protein and a low carbohydrate content (71.9% and 9.6% of biomass dry weight, respectively) were measured in cells under this condition. It is concluded that *K. marxianus* aligns with the so-called aerobic-respiring or Crabtree-negative yeasts. Furthermore, it has one of the highest growth rates among yeasts, and a high capacity of converting sugar into biomass, even when carbon is not the limiting nutrient. These results provide useful data regarding the future application of *K. marxianus* in processes aimed at the production of biomass-linked compounds, with high yields and productivities.

Introduction

The number of described yeast species increases every year and in the last compendium edited by Kurtzman & Fell (1998), more than 700 different species belonging to 100 genera are reported. In spite of this immense spectrum, industrial or biotechnological applications are still limited to a very small number of species, mainly belonging to the genera *Saccharomyces*, *Kluyveromyces*, *Hansenula*, *Pichia* and *Yarrowia* (de Winde, 2003). Among these, *Saccharomyces cerevisiae* occupies an outstanding position, as it is not only the most widely employed yeast but also the most utilized microorganism in the biotechnological industry. Applications involving this yeast are diverse and vary from the traditional processes for food and beverage production, to the more advanced processes which aim at producing pharmaceutical compounds. In the specific case of ethanol production, *S. cerevisiae* can hardly be beaten by any other yeast, owing to its high capacity of converting sugars into ethanol, under both anaerobic and aerobic conditions. By contrast, *S. cerevisiae* might not be the most suitable organism with regard to biotechnological processes aimed

at producing a compound whose titer is linked to biomass formation. This is mainly due to the strong Crabtree-positive characteristic of *S. cerevisiae*, which requires a controlled supply of the carbon source to avoid fermentative metabolism, the occurrence of which is highly undesirable in biomass-directed applications. This obviously poses a limit on the productivity of such a process. Furthermore, in the case of heterologous protein production, *S. cerevisiae* is known to have a tendency for hypermannosylation (Merico *et al.*, 2004).

Among the non-*Saccharomyces* or nonconventional yeasts (Wolf *et al.*, 2003) with potential for industrial applications are those belonging to the genus *Kluyveromyces*. In particular, *Kluyveromyces lactis* was the first yeast after *S. cerevisiae* to be given GRAS status (generally recognized as safe) (Bonekamp & Oosterom, 1994), an important prerequisite for many applications. This species has been used industrially for the production of enzymes such as lactase and chymosin (van den Berg *et al.*, 1990; Swinkels *et al.*, 1993). These applications certainly benefit from the vast amount of research that has already been carried out on *K. lactis* in recent decades, in terms of both its physiology and its genetics, which culminated with the completion of its genome sequencing (Dujon *et al.*, 2004). In terms of its energetic metabolism, this species has a Crabtree-negative or an aerobic-respiring characteristic, in contrast to *S. cerevisiae*. *Kluyveromyces lactis* is easily amenable to genetic manipulation and suitable genetic tools have already been made available for this yeast (Breunig & Steensma, 2003; Wolf *et al.*, 2003). *Kluyveromyces marxianus*, which is closely related to *K. lactis*, has received much less attention from the scientific community, in spite of some very interesting characteristics it possesses. Among others, it can utilize a vast range of substrates, it grows more rapidly than *K. lactis*, even at temperatures as high as 40 °C (Rouwenhorst *et al.*, 1988; Steensma *et al.*, 1988), and it also has GRAS status, which makes it particularly suitable for the production of pharmaceuticals and food-grade proteins (Hensing *et al.*, 1994; 1995).

The great majority of studies published on *K. marxianus* have explored potential applications of this organism without investigating what takes place at the intracellular level. Usually, the yeast cells are cultivated on a natural (complex) substrate and measurements are carried out in such a way that only the concentration of one substrate and product, in addition to the cell concentration, are determined. Carbon balances are very rarely closed, giving only a rough macroscopic picture of the cellular reactions. In spite of this, these studies have shown that *K. marxianus* is very versatile and could be economically explored for a very wide range of applications. Examples include the production of ethanol (Singh *et al.*, 1998; Kourkoutas *et al.*, 2002), cell protein (Kim *et al.*, 1998; Grba *et al.*, 2002; Schultz *et al.*, 2006), bioingredients (Belem & Lee, 1998), enzymes, such as inulinase (Rouwenhorst *et al.*, 1988; Hensing *et al.*, 1994, 1995; Passador-Gurgel *et al.*, 1996), lactase (Rech *et al.*, 1999; Martins *et al.*, 2002), pectinase (Cruz-Guerrero *et al.*, 1999), lipase (Deive *et al.*, 2003), as well as aroma compounds (Wittmann *et al.*, 2002; Etschmann *et al.*, 2004), and its use as baker's yeast (Caballero *et al.*, 1995) and as an anticholesterolemic agent (Yoshida *et al.*, 2004).

With the aim of increasing our knowledge on the macroscopic physiology of *K. marxianus*, we performed batch and continuous bioreactor cultivations of this yeast under well-defined conditions, and carried out measurements which allowed us quantitatively to describe growth, substrate consumption, metabolite formation, biomass composition and respiratory parameters, when glucose is used as the sole carbon source.

Material and methods

Strain, maintenance, and preculture

Kluyveromyces marxianus ATCC 26548 (= CBS 6556, NCYC 2597, NRRLy 7571) was purchased from ATCC in lyophilized form. Subsequently, it was cultured in YPD medium (yeast extract, 10 g L^{-1}; peptone, 20 g L^{-1}; glucose, 20 g L^{-1}) until late exponential phase, glycerol was added to a final concentration of 15% (v/v) and 1-mL aliquots of this culture were stocked frozen at − 80 °C. For the preparation of experiments, a frozen stock vial was used to inoculate a yeast and malt extract agar (YMA) plate, which was stored at 4 °C for up to 3 months. Inoculum was prepared by transferring cells from the YMA plate to 5 mL of liquid YPD medium held in a 50-mL baffled Erlenmeyer flask. After 8 h growth on an orbital shaker (200 r.p.m.) at 30 °C, cells were centrifuged, washed twice with 5 mL NaCl 0.9 M solution and resuspended in mineral medium.

Mineral medium for precultures and bioreactor cultivations

The mineral medium (Verduyn *et al.*, 1992) contained, per liter of distilled water: $(NH_4)_2SO_4$, 5.0 g; KH_2PO_4, 3.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; trace elements (EDTA, 15 mg; $ZnSO_4 \cdot 7H_2O$, 4.5 mg; $MnCl_2 \cdot 2H_2O$, 0.84 mg; $CoCl_2 \cdot 6H_2O$, 0.3 mg; $CuSO_4 \cdot 5H_2O$, 0.3 ; $Na_2MoO_4 \cdot 2H_2O$, 0.4 ; $CaCl_2 \cdot 2H_2O$, 4.5 mg; $FeSO_4 \cdot 7H_2O$, 3.0 mg; H_3BO_3, 1.0 mg; KI, 0.1 mg); silicone antifoam, 0.05 mL. It was adjusted to pH 6.0 with KOH before autoclaving (121 °C, 20 min). The medium was cooled to room temperature and a filter-sterilized solution of vitamins prepared in demineralized water was added, to a final concentration, per liter, of: d-biotin, 0.05 mg; calcium pantothenate, 1.0 mg; nicotinic acid, 1.0 mg; *myo*-inositol, 25 mg; thiamine HCl, 1.0 mg;

pyridoxin HCl, 1.0 mg; and para-aminobenzoic acid, 0.20 mg. Glucose was sterilized separately and added to a final concentration of 10 g L^{-1}.

Bioreactor cultivations

Cultivations were performed in a Vario 1000 bioreactor (Meredos, Bovenden, Germany), and started by adding a certain volume of the resuspended preculture, so that the initial cell concentration ($OD_{600\,nm}$) in the bioreactor was 0.001.

Batch cultivation conditions were 30 °C, 100 mL working volume with pH controlled at 5.0 by automatic addition of 0.1 M KOH. The culture was sparged with air at a flow rate of 6 L h^{-1} (1 v.v.m.) and stirred at 1000 r.p.m. The dissolved oxygen concentration was continuously monitored with an oxygen probe and was always above 50% saturation.

Continuous cultivations were preceded by a batch cultivation, under the same temperature, pH and stirring conditions as described above. After glucose had been exhausted, which was verified online by the rapid increase of the dissolved oxygen concentration and offline by constant biomass concentration measurements, the cultivations were switched to continuous mode. Aeration was performed at a flow rate of 6 L h^{-1}. The reactor volume was kept constant using a continuously operating effluent pump coupled to a mechanical drain. Steady state was verified after at least five volume changes had taken place since the last modification in the cultivation conditions. Five samples were then withdrawn, with a 1-h interval between two consecutive samples, and checked for constant biomass concentration.

Samples (0.2 mL each) were collected regularly from the reactor outlet in centrifuge tubes in an ice-water bath; 0.1 mL was used for $OD_{600\,nm}$ measurement, after appropriate dilution. The remaining 0.1 mL was centrifuged at 4 °C, 16 000 ***g*** for 5 min (Biofuge Pico, Heraeus, Hanau, Germany). The supernatant was frozen at − 80 °C and later used for determining the concentration of extracellular metabolites. For the batch cultures and for each final steady-state sample (before the dilution rate was changed), samples (approximately half of the fermentor working volume) were withdrawn directly from the cultivation medium in an ice-water bath, weighed and centrifuged (4 °C, 16 000 ***g*** for 5 min). The cell pellet was resuspended in MilliQ water and centrifuged again. The resulting pellet was resuspended in MilliQ water, in such a way that the same weight initially removed from the reactor was obtained. Finally, the samples were frozen at − 80 °C and later used for the determination of biomass composition.

Gas analysis

Composition of inlet and outlet gases was measured online by MS (Omnistar, Inficon, Liechtenstein). The two gas streams were alternately analysed resulting in a time interval of about 2 min between two consecutive measurements. The mass spectrometer was calibrated 24 h before the start of cultivation using synthetic air containing a defined concentration of carbon dioxide (0.057 ± 0.005%). OUR and CER were calculated from oxygen and carbon dioxide content in the inlet and exhaust gases, respectively. Nitrogen was taken as inert gas in the balance equation (Heinzle & Dunn, 1991).

Determination of biomass and extracellular metabolite concentration

Biomass concentration in terms of grams dry weight per culture volume was indirectly determined via OD measurements performed with a spectrophotometer (Ultrospec 2000; Pharmacia, Freiburg, Germany) at 600 nm. For this purpose, the measured absorbance values were converted into mass values using a linear relationship of 0.57 OD units per gram dry cell mass. For the last samples at steady state during chemostats, which were used for the determination of biomass composition, dry cell weight was determined directly using 0.45-μm membrane filters and a microwave oven (180 W, 15 min) (Olsson & Nielsen, 1997).

Glucose and organic acids were separated by HPLC on an Aminex HPX-87H ion-exclusion column (300 × 7.8 mm; Bio-Rad, Hercules, CA) at 45 °C, using 5 mM H_2SO_4 as mobile phase at a flow rate of 0.8 mL min^{-1}. These compounds were detected by a UV-absorbance detector at 210 nm (HPLC 535; Kroma System, Kontron Instruments, Neufahrn, Germany) connected in series with an RI detector (ERC-7515A; ERC Inc., Alteglofsheim, Germany). Ethanol was quantified enzymatically using a Sigma Diagnostic Kit (St Louis, MO).

Extracellular amino acids were separated by HPLC using a Grom-Sil OPA-3 column (125 × 4 mm; Grom-Analytik, Herrenberg, Germany) and quantified as isoindol-derivatives by fluorescence (Agilent Series 1100; Agilent Technologies, Waldbronn, Germany) with excitation at 330 nm and emission at 450 nm.

Determination of parameters during the exponential growth phase (EGP)

The EGP was identified as the linear region on an ln (X) vs. time plot for batch cultivation data. The maximum specific growth rate (μ_{max}) was determined as the slope of this linear region. The biomass yield on substrate ($Y_{X/S}$) was determined as the slope of the line on an X vs. S plot, exclusively including points belonging to the EGP. The specific rate of substrate consumption (μ_S) was calculated according to the following equation:

$$\mu_S = \mu_{max}/Y_{X/S}$$

where μ_{max} = maximum specific growth rate (h^{-1}); X = biomass concentration in the bioreactor (g DW L^{-1}); μ_S = specific rate of substrate consumption during the EGP [g (g DW h)$^{-1}$]; S = substrate concentration in the bioreactor (g L^{-1}); and $Y_{X/S}$ = biomass yield on substrate during the EGP (g DW g^{-1}); DW = dry cell weight.

Determination of parameters during continuous cultivations

Specific growth rate (μ), specific rate of substrate consumption (μ_S) and biomass yield on glucose ($Y_{X/S}$) were calculated as follows:

$$\mu = D$$

$$\mu_S = \frac{1}{X} D(S_F - S)$$

$$Y_{X/S} = \frac{X}{S_F - S}$$

where μ = specific growth rate (h^{-1}); X = biomass concentration in the bioreactor (g DW L^{-1}); D = dilution rate (h^{-1}); μ_S = specific rate of substrate consumption [g (g DW h)$^{-1}$]; S_F = substrate concentration in the feeding medium (g L^{-1}); S = substrate concentration in the bioreactor (g L^{-1}); and $Y_{X/S}$ = biomass yield on glucose.

Biomass composition

Cell protein content was measured by an adaptation of the method described by Verduyn *et al.* (1990). Carbohydrate content was determined using the phenol–sulfuric acid method as described by Dubois *et al.* (1956). Lipids were extracted from cells using a mixture of chloroform and methanol, according to Bligh & Dyer (1959). RNA was quantified according to Benthin *et al.* (1991). DNA measurement was by Burton's method as described by Herbert *et al.* (1971). Ash was expressed as the percentage of residue remaining after dry oxidation, according to the American Society for Testing and Materials (2003).

Results

Growth, CO_2 formation, and consumption of glucose and O_2

The main cultivation parameters obtained in this study during aerobic growth of *K. marxianus* ATCC 26548 in batch and chemostat cultures on glucose as the sole C-source are given in Table 1. During the EGP of two independent batch cultivations (Fig. 1), the maximum specific growth rate (μ_{max}) of this strain was calculated as $0.56 \pm 0.02\ h^{-1}$. Also during the EGP, the biomass yield on glucose ($Y_{X/S}$) was estimated as 0.51 ± 0.02 g DW (g glucose)$^{-1}$, and the specific rate of glucose consumption (μ_S) was 1.095 ± 0.005 (g g DW h^{-1}).

Steady states in aerobic continuous cultures with *K. marxianus* growing on glucose as the sole carbon source were obtained at dilution rates of 0.1, 0.25 and 0.5 h^{-1}, with 1 v.v.m. air sparging. The biomass yield on glucose was approximately the same at 0.1, 0.25 and 0.5 h^{-1} (Table 1). At all three dilution rates, the respiratory quotient (RQ) was close to unity, indicating that the metabolism of *K. marxianus* was purely or almost exclusively respiratory. The specific rates of CO_2 production (qCO_2) and O_2 consumption (qO_2) increased with the dilution rate (Table 1).

An additional steady state was achieved at 0.1 h^{-1} with 2.5 v.v.m. air sparging. From the data obtained, it is difficult to confirm whether qO_2 and qCO_2 during this cultivation were slightly lower than the corresponding parameters obtained at the same dilution rate, but with 1 v.v.m. air sparging, or whether the values can be considered the same in both cultivations. Regardless, the RQ for the 0.1 h^{-1} and 2.5 v.v.m. chemostat was also very close to unity. However, the biomass yield on glucose was slightly lower at this steady state, as compared with that obtained at the remaining three cultivations, which were all carried out with 1 v.v.m. air sparging.

Metabolite formation and carbon balances

Metabolites were formed to a very low extent under the conditions employed (Tables 2 and 3), totalling always $< 3\%$ of the consumed carbon, except for the chemostat at 0.5 h^{-1}, in which total metabolites corresponded to 6% of the consumed carbon. However, as the fed glucose was not completely consumed by the yeast cells in the latter experiment, this was not a carbon-limited culture, which is a different physiological situation as compared with the other cultures performed in this work. In general, metabolite formation increased with dilution rate. Interestingly, at $D = 0.1\ h^{-1}$ and 2.5 v.v.m., metabolite formation was higher than for the culture at the same dilution rate and 1v.v.m. air sparging, and also higher for some metabolites as compared with the culture at 0.25 h^{-1}. In general, acetate, pyruvate and 2-oxoglutarate were the organic acids present in highest concentrations (Table 2; Fig. 2).

The excess of residual glucose (7.25 g L^{-1}) at the high dilution rate ($D = 0.5\ h^{-1}$) did not provoke significant alcoholic fermentation, which is in accordance with the Crabtree-negative character of *K. marxianus*. Regardless, the amount of ethanol produced increased slightly with the dilution rate.

Table 3 illustrates the recovery of carbon in the excreted products. Carbon recovery in biomass remained around 0.59 C-mol biomass per C-mol glucose for all cultivations,

Table 1. Main growth parameters obtained in this work for *Kluyveromyces marxianus* compared with those obtained for other yeast strains

Yeast strain	Cultivation mode	μ (h^{-1})	μ_S [g (g DW h^{-1})]	DT (h)	$Y_{X/S}$ (g DW g^{-1})	*q*O2 (mmol g^{-1} h^{-1})	*q*CO2 (mmol g^{-1} h^{-1})	RQ	Reference
K. marxianus ATCC 26548	Batch*	μmax = 0.56 ± 0.02	1.095 ± 0.005	1.24	0.51 ± 0.02	11.05 ± 1.03	12.06 ± 0.55	1.09	This work
(= CBS 6556)	Continuous†,‡	*D* = 0.1	0.22	6.93	0.45 ± 0.00	2.67 ± 0.07	2.69 ± 0.08	1.01	
	Continuous†	*D* = 0.1	0.20	6.93	0.49 ± 0.00	2.87 ± 0.08	2.82 ± 0.09	0.98	
	Continuous†	*D* = 0.25	0.52	2.77	0.48 ± 0.00	6.65 ± 0.06	7.30 ± 0.07	1.10	
	Continuous†	*D* = 0.5	1.05	1.39	0.48 ± 0.00	11.09 ± 0.19	11.50 ± 0.12	1.04	
K. marxianus CBS 6556	Batch	μmax = 0.44 ± 0.03	0.90	1.57	0.49	–	13.46	–	Bellaver *et al*. (2004)
	Continuous§	*D* = 0.1	–	6.93	–	–	4.0 ± 0.3	–	Rouwenhorst *et al*. (1991)
	Continuous¶	*D* = 0.1	–	6.93	–	3.1	–	–	Verduyn *et al*. (1992)
	Continuous‖	*D* = 0.1	0.23	6.93	0.43	–	–	–	Postma & van den Broek (1990)
	Continuous‖	*D* = 0.2	0.46	3.46	0.43	–	–	–	
	Continuous**	*D* = 0.2	0.42	3.46	0.48	–	–	–	Hensing *et al*. (1994)
	Continuous††	*D* = 0.2	0.5	3.46	0.40	–	–	–	
K. lactis CBS 2359	Continuous	*D* = 0.1	0.21	6.93	0.48	3.7	3.7	1	Kiers *et al*. (1998)
	Continuous	*D* = 0.2	0.41	3.46	0.49	6.2	6.2	1	
	Continuous	*D* = 0.4	0.82	1.73	0.49	11.3	11.3	1	
S. kluyvery Y708	Continuous	*D* = 0.1	0.22	6.93	0.46	4.2	4.1	0.98	Møller *et al*. (2002)
	Continuous	*D* = 0.3	0.62	2.31	0.48	9.5	10	1.05	
	Continuous	*D* = 0.54	1.08	1.28	0.50	13.6	15	1.1	
	Continuous	*D* = 0.63	2.62	1.10	0.24	6.8	20.4	3.0	
S. cerevisiae CBS 8066	Continuous‡‡	*D* = 0.1	0.20	6.93	0.51	2.5	2.7	1.08	Bruinenberg *et al*. (1986), Postma *et al*. (1989a, b), van Dijken *et al*. (1993)
	Continuous‡‡	*D* = 0.3	0.60	2.31	0.50	7.5	8.5	1.1	
	Continuous‡‡	*D* = 0.4	1.82	1.73	0.22	9	20.5	2.3	
S. cerevisiae LBGH-1022	Continuous	*D* = 0.1	0.21	2.77	0.49	–	–	1.07	Furukawa *et al*. (1983)
		D = 0.25	0.50	2.77	0.50	7.1	7.6	1.07	
	Continuous	*D* = 0.3	1.41	2.31	0.21	3.4	18.5	5.44	
S. cerevisiae CEN.PK 113-7D	Continuous	*D* = 0.1	0.21	6.93	0.48	2.7	2.8	1.04	van Hoek *et al*. (1998b)
	Continuous	*D* = 0.25	0.20	2.77	0.49	7.0	7.3	1.04	
	Continuous	*D* = 0.38	2.37	1.82	0.16	3.9	21	5.38	
S. cerevisiae DS2891	Continuous	*D* = 0.1	0.21	6.93	0.48	2.5	2.7	1.08	van Hoek *et al*. (1998a)
	Continuous	*D* = 0.25	0.52	2.77	0.48	7.0	7.5	1.07	
	Continuous	*D* = 0.4	2.00	1.73	0.20	3.7	18.9	5.11	

*Average and SD from two independent cultivations.

†Average and SD calculated from five samples obtained at 1-h intervals during each steady state.

‡This cultivation was carried out under the same conditions as the other experiments in this work (30 °C, pH 5.0, glucose 10 g L^{-1}), except for aeration, which was 2.5 v.v.m., instead of 1 v.v.m.

§Sucrose 5 g L^{-1} (40 °C).

¶37 °C.

‖40 °C.

**Sucrose 5 g L^{-1} (30 °C).

††Sucrose 5 g L^{-1} (40 °C).

‡‡Glucose 15 g L^{-1}.

DT, doubling time; $Y_{X/S}$, biomass yield on substrate; v.v.m., air volume per culture volume per minute.

Some data from other authors presented in this table were obtained from graphics or calculated.

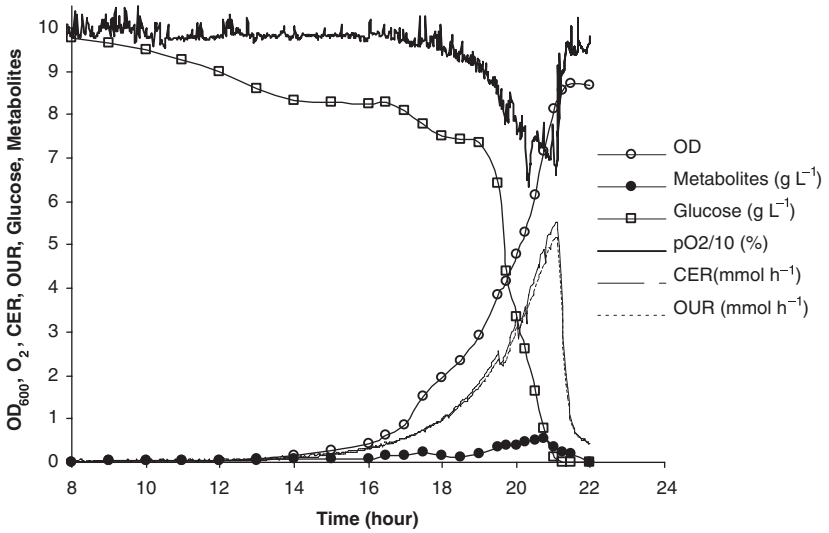

Fig. 1. Kinetics of growth, total metabolite formation, glucose consumption, dissolved oxygen concentration, carbon dioxide evolution rate and oxygen uptake rate during batch cultivation of *Kluyveromyces marxianus* ATCC 26548.

except for the cultivation at 0.1 h^{-1} and 2.5 v.v.m., in which it was slightly lower (Table 3). In terms of carbon recovery in the produced CO_2, lowest values were for the cultivation at 0.5 h^{-1}, which was not a carbon-limited cultivation. In all cultivations performed, carbon balances closed within 95–100% (Table 3).

Biomass composition and extracellular amino acids

The biomass composition of *K. marxianus* was evaluated for protein, carbohydrate, lipid, RNA, DNA and ash as percentage of dry weight for the batch and continuous cultures, as summarized in Table 4. A considerable increase in protein content was observed with increasing growth rate. The carbohydrate content was inversely related to the protein content. The lipid content was generally constant among the experimental conditions, except for the cultivation at 0.1 h^{-1} and 2.5 v.v.m., in which it was higher. The RNA content increased with the specific growth rate. The DNA content was very low at all cultivation conditions investigated. The ash content also remained fairly constant, but perhaps with a slight tendency to increase with the specific growth rate (Table 4).

Extracellular amino acids were measured in the culture supernatants. The most prevalent extracellular amino acid was tryptophane (93.6% of total amino acids). However, extracellular amino acids were present in insignificant amounts during batch exponential growth, totalling 0.015 C-mmol C-mol substrate^{-1}. Tryptophane was also the predominant amino acid in supernatants of continuous cultures [80.4%, 83.5%, 89.4% and 93.9% of the total amino acids for $D = 0.1\ h^{-1}$ (2.5 v.v.m.), 0.1 h^{-1}, 0.25 h^{-1} and 0.5 h^{-1}, respectively]. However, they were only present in insignificant concentrations (0.0025 ± 0.0015 C-mmol C-mol substrate^{-1}).

Discussion

Growth, CO_2 formation and consumption of glucose and O_2

Conflicting data plus the scarcity of information maintain doubts regarding the maximum specific growth rate of *K. marxianus* CBS 6556. Rouwenhorst *et al.* (1988) gave values as high as 0.69 h^{-1} for growth at 33 °C on mineral medium with glucose as the sole carbon source. At 40 °C, the specific growth rate was even higher (0.86 h^{-1}) (results from shake flask cultures). Hoekstra *et al.* (1994) affirmed that the maximum specific growth rate of this strain is as high as 1.1 h^{-1}, without specifying the cultivation conditions and the source from which this value was obtained. However, their own measurements resulted in a μ_{max} value of 0.70 h^{-1} for batch growth on YPD medium (with glucose as a carbon source), at 37 °C. These same authors established a steady-state continuous cultivation at a dilution rate as high as 0.85 h^{-1}. Finally, recently reported values of μ_{max} from our own group were as low as $0.44 \pm 0.03\ h^{-1}$ (Bellaver *et al.*, 2004), which was calculated from triplicate experiments. As the value of $0.56 \pm 0.02\ h^{-1}$ reported in the present work is a result of duplicate experiments, the difference observed is not due to measurement errors but rather to a physiological difference between the strains used in the different studies. In fact, the strains were not obtained from the same source and were not cultivated in the same laboratory. Taking these facts into account and the much higher values reported by other groups for the same strain, it is tempting to speculate that the history of strain preservation and manipulation

Table 2. Metabolite production (C-mmol C-mol substrate^{-1}) from different *Kluyveromyces marxianus* cultivations

Cultivation mode	Culture (h^{-1})	Citrate	2-Oxoglutarate	Pyruvate	Succinate	Lactate	Fumarate	Acetate	Glycerol	Ethanol
Batch*	$\mu_{max} = 0.56 \pm 0.02$	0.82	17.12	20.52	1.74	1.53	0.81	22.15	4.55	10.12
Continuous†,‡	$D = 0.1$	0 ± 0	1.84 ± 0.24	7.98 ± 1.27	0.21 ± 0.06	4.22 ± 2.99	0 ± 0	11.18 ± 1.86	0 ± 0	1.87 ± 0.48
Continuous†	$D = 0.1$	0 ± 0	0.03 ± 0.07	0.33 ± 0.24	0 ± 0	0 ± 0	0 ± 0	1.33 ± 0.17	0 ± 0	0 ± 0
Continuous†	$D = 0.25$	0 ± 0	0.11 ± 0.16	0.50 ± 0.16	0 ± 0	0.08 ± 0.18	0.03 ± 0.06	1.40 ± 0.16	0.57 ± 0.69	2.00 ± 0.07
Continuous†	$D = 0.5$	0 ± 0	14.12 ± 3.07	11.50 ± 2.56	2.34 ± 1.46	0.29 ± 0.13	3.32 ± 0.24	15.25 ± 2.81	0 ± 0	8.52 ± 0.22

*Data refer to the instant when total metabolite concentration was the highest (20.5 h, Fig. 1). Average and SD calculated from two independent cultivations.

†Average and SD calculated from five samples obtained at 1-h intervals during each steady state.

‡This cultivation was carried out under the same conditions as the other experiments in this work (30 °C, pH 5.0, glucose 10 g L^{-1}), except for aeration, which was 2.5 v.v.m., instead of 1 v.v.m.

plays a major role in the physiology of *K. marxianus*. As it is known that this species presents a high level of intraspecific polymorphism (Belloch *et al.*, 1998), it may have a high mutation rate, which results in rapid and unexpected evolution during the propagation steps that are involved from stock until the cultivation in different laboratories.

It is difficult to compare our data from continuous cultivations with the results reported by other authors because experiments were performed at different conditions with regard to the nature and the concentration of the sugar used as a carbon source, as well as the cultivation temperature. It is evident that the biomass yield on substrate is influenced by the temperature, with lower conversions at higher temperatures (Hensing *et al.*, 1994). The value obtained in the present work for growth at 0.1 h^{-1} and 30 °C compares well with that reported by Hensing *et al.* (1994) for growth of the same strain on sucrose at 0.2 h^{-1} at the same temperature (Table 1). However, Postma & van den Broek (1990), who cultivated the same strain under analogous conditions, obtained biomass yields of 0.43 g DW g^{-1} glucose for steady-state chemostats at 0.1 and 0.2 h^{-1}, which are lower than the values obtained in the present work (Table 1).

The low biomass concentration of 1.31 g DW L^{-1} and the high residual glucose concentration (7.25 g L^{-1}) obtained at the dilution rate of 0.5 h^{-1} (Table 3) indicate that a nutrient limitation different from that of carbon limitation is occurring under these conditions. Analogous observations are reported by Kiers *et al.* (1998), who describe a high residual glucose concentration and a biomass formation not proportional to the glucose concentration in the feeding medium, during a continuous culture of *K. lactis* at a high dilution rate (0.4 h^{-1}). The authors later confirmed that this physiological behavior was due to a limitation of nicotinic acid in the feeding medium. Interestingly, in the present work, in spite of this nutrient limitation, the biomass yield on glucose and the respiratory quotient remained at similar levels, as compared with the corresponding values obtained during the chemostat at 0.1 h^{-1}. It was not the purpose of this study to investigate which nutrient limitation occurs when *K. marxianus* ATCC 26548 is grown in a chemostat at 0.5 h^{-1}. However, it might be that the limiting nutrient is not nicotinic acid, but thiamine, as α-ketoglutarate accumulated in these cultures, a phenomenon that has already been described for yeasts growing under thiamine limitation (Chernyavskaya *et al.*, 2000).

The respiratory parameters obtained in this study were compared with data previously reported by other authors at similar conditions, as well as with data obtained for *K. lactis*, *Saccharomyces kluyveri* and four *S. cerevisiae* strains (Table 1, Fig. 3). It can be observed that up to medium-range dilution rates, aerobic-fermenting (*S. cerevisiae*) and aerobic-respiring (*S. kluyveri*, *K lactis* and *K. marxianus*) yeasts show

Table 3. Carbon balance (C-mol C-mol substrate^{-1}) and recovery (%) of different *Kluyveromyces marxianus* cultivations

Cultivation mode	Culture (h^{-1})	Glucose	Biomass*	Metabolites	CO_2	Remaining glucose	Carbon recovered (%)
Batch[§]	$\mu_{max} = 0.56 \pm 0.02$	−1	0.612 ± 0.007	0 ± 0	0.352 ± 0.002	0 ± 0	96.41 ± 1.40
Continuous[‡,†]	$D = 0.1$	−1	0.555 ± 0.010	0.029 ± 0.004	0.367 ± 0.002	0 ± 0	95.02 ± 1.36
Continuous[‡]	$D = 0.1$	−1	0.594 ± 0.012	0.002 ± 0.000	0.401 ± 0.002	0 ± 0	99.68 ± 1.53
Continuous[‡]	$D = 0.25$	−1	0.581 ± 0.009	0.005 ± 0.001	0.416 ± 0.003	0 ± 0	100.23 ± 1.60
Continuous[‡]	$D = 0.5$	−1	0.582 ± 0.009	0.056 ± 0.008	0.330 ± 0.004	-0.725 ± 0.002	96.81 ± 1.49

*For the carbon content in biomass, a 24.6 g (C-mol)$^{-1}$ biomass relationship was used in all cases (Stephanopoulos *et al.*, 1998).

†This cultivation was carried out under the same conditions as the other experiments in this work (30 °C, pH 5.0, glucose 10 g L^{-1}), except for aeration, which was 2.5 v.v.m., instead of 1 v.v.m.

‡Average and SD calculated from five samples obtained at 1-h intervals during each steady state.

§Data refer to the end of the culture (22 h, Fig. 1). Average and SD calculated from two independent cultivations.

Table 4. Composition of biomass during cultivations of *Kluyveromyces marxianus* ATCC 26548[†]

Yeast strain	Cultivation mode	Culture (h^{-1})	Protein	Carbohydrate	Lipid	RNA	DNA	Ash	Total	Reference
K. marxianus ATCC	Batch*	$\mu_{max} = 0.56 \pm 0.02$	54.6 ± 1.5	26.5 ± 0.8	5.2 ± 0.2	10.7 ± 0.1	0.7 ± 0.1	3.0 ± 0.2	100.7	This work[†]
26548 (= CBS 6556)	Continuous*,‡	$D = 0.1$	35.9 ± 1.3	51.1 ± 1.0	7.2 ± 0.1	5.1 ± 0.2	0.3 ± 0.1	1.2 ± 0.5	100.8	
	Continuous*	$D = 0.1$	37.0 ± 1.5	49.5 ± 1.1	5.1 ± 0	4.9 ± 0.3	0.2 ± 0.1	2.6 ± 0.1	99.3	
	Continuous*	$D = 0.25$	52.9 ± 1.0	31.3 ± 0.9	5.1 ± 0	7.8 ± 0.3	0.5 ± 0	2.3 ± 0.2	99.9	
	Continuous*	$D = 0.5$	71.9 ± 2.7	9.6 ± 0.8	5.1 ± 0	10.6 ± 0.1	0.6 ± 0.1	2.6 ± 0.1	100.4	
K. marxianus FII 510700	Batch	–	56	26	–	10	2.7	–	–	Lukondeh *et al.* (2003)
K. marxianus LG	Batch	–	54	–	–	–	–	–	–	Guiraud *et al.* (1981)
S. cerevisiae (in general)	Batch[§]	$\mu_{max} = 0.37$	51	27	7	11	–	4	100	Gombert *et al.* (2001)
	Continuous[§]	$D = 0.1$	42	39	7	7	–	4	100	
S. cerevisiae LBGH-1022	Continuous[¶]	$D = 0.2$	40	50	8	6.5	0.3	–	–	Furukawa *et al.* (1983)
	Continuous[¶]	$D = 0.25$	43	42	6.5	7.5	0.3	–	–	
	Continuous[¶]	$D = 0.3$	48	44	3.2	8.5	0.3	–	–	
S. cerevisiae CBS 8066	Continuous[‖]	$D = 0.1$	45	40.7	2.9	6.3	0.4	5	101.4	Nissen *et al.* (1997)
	Continuous[‖]	$D = 0.2$	50	32.7	3	8.2	0.4	5	100.6	
	Continuous[‖]	$D = 0.3$	55.5	25.2	3.8	10.1	0.5	5	101.2	
	Continuous[‖]	$D = 0.4$	60.1	17	3.4	12.1	0.6	5	100.2	

*Average and SD calculated from two independent cultivations, two samples taken from each cultivation (batches). For the chemostats, average and SD were calculated from two analyses carried out on the last of the five samples withdrawn during steady state. In all samples, carbohydrates were analysed in triplicate.

†Data are given in percent (w/w).

‡This cultivation was carried out under the same conditions as the other experiments in this work (30 °C, pH 5.0, glucose 10 g L^{-1}), except for aeration, which was 2.5 v.v.m., instead of 1 v.v.m.

§Compendium of data from diverse authors.

¶Dissolved oxygen > 1 mg L^{-1}.

‖Anaerobic growth; total also includes free amino acids (%).

approximately the same behavior and values for CO_2 production and O_2 consumption (Fig. 3). However, the aerobic-fermenting strains maintain their fully respiratory metabolism only up to dilution rates of 0.28 h^{-1} (*S. cerevisiae* DS28911; van Hoek *et al.*, 1998b) and 0.38 h^{-1} (*S. cerevisiae* CBS 8066; Postma *et al.*, 1989b). From this point, the production of CO_2 becomes much higher and the consumption of O_2 much lower in aerobic-fermenting strains than in aerobic-respiring strains.

According to Møller *et al.* (2002), values of qO_2 are strain dependent. In their work with *S. kluyveri*, a maximum qO_2 of 13.6 mmol [(g DW)$^{-1}$ h^{-1}] at $D = 0.54$ h^{-1} was observed

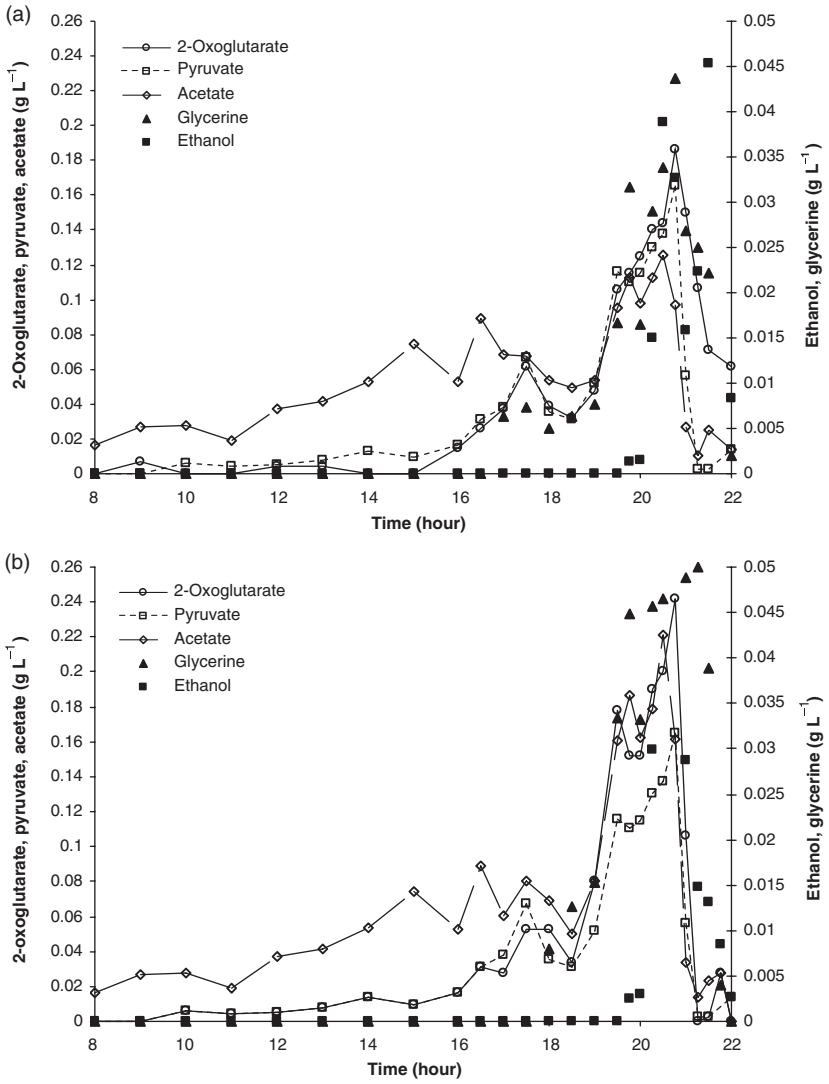

Fig. 2. Kinetics of metabolite formation during batch cultivation of *Kluyveromyces marxianus* ATCC 26548. Each graph corresponds to a repetition of the same experiment.

(Table 1). Above this value, the quotient between the rates of CO_2 formation and O_2 consumption (RQ) increased far above 1, indicative of fermentative metabolism setting in. For *K. lactis* (Kiers *et al.* 1998) and *K. marxianus* (this study), maximum qO_2 values of 11.3 and 11.1 mmol $[(g\,DW)^{-1}\,h^{-1}]$ were obtained at dilution rates of 0.4 and $0.5\,h^{-1}$, respectively. These values are very close to each other, slightly lower than the maximum values indicated above for *S. kluyveri*, and higher than those observed for *S. cerevisiae* at any condition (except for the OB58066 strain), which is a natural reflection of the higher respiratory capacity of aerobic-respiring yeasts as compared with *S. cerevisiae*. Continuous cultures of *K. marxianus* never showed any indication of oscillations, such as those observed for *S. cerevisiae* under aerobic-respiratory conditions (von Meyenburg, 1969; Heinzle *et al.*, 1982).

Metabolite production and carbon balances

Kluyveromyces marxianus ATCC 26548, despite its Crabtree-negative characteristic, was reported to have a strong tendency to produce pyruvate and acetate when exposed to excess sugar (Hensing *et al.*, 1994). Besides these two compounds, we also observed the presence of 2-oxoglutarate in the supernatants of samples taken from the chemostat at $0.5\,h^{-1}$ and from the chemostat at $0.1\,h^{-1}$ and 2.5 v.v.m. As already discussed above, the former is possibly a consequence of thiamine limitation. However, the formation of 2-oxoglutarate at $0.1\,h^{-1}$ and 2.5 v.v.m., at amounts equal to that of ethanol in terms of C-mol per C-mol consumed substrate, is quite remarkable as it is not a product commonly observed during culture of yeasts. Furthermore, lactate is also formed to some extent under this condition.

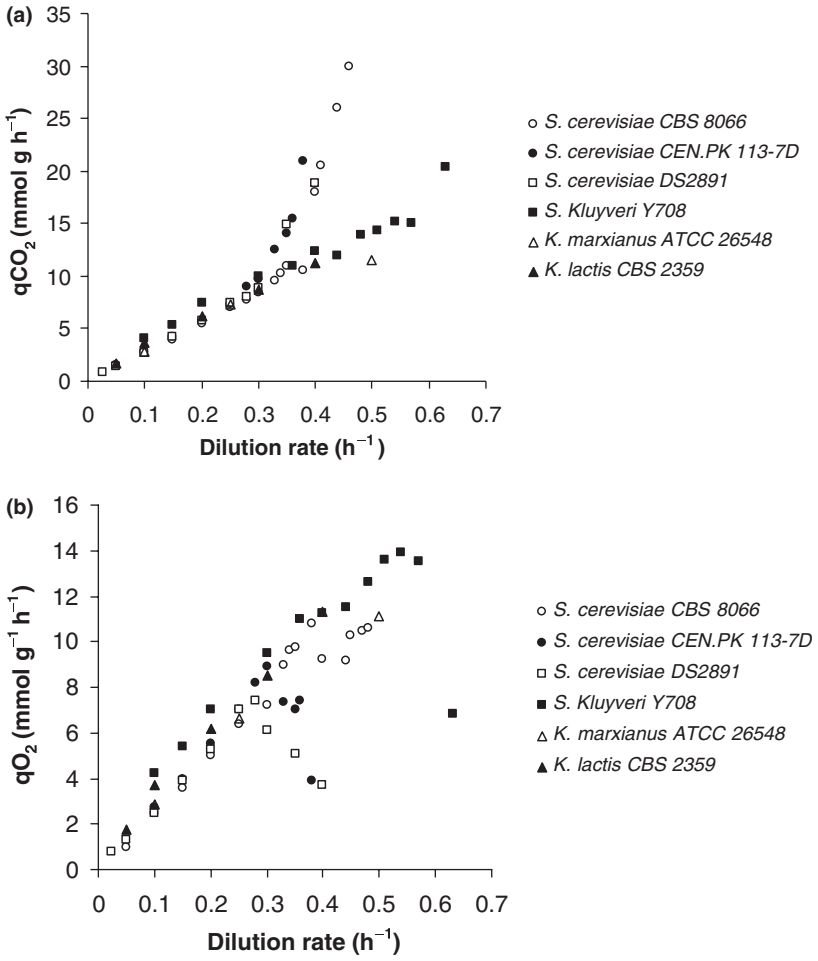

Fig. 3. Specific CO_2 production rate (a) and specific O_2 consumption rate (b) of several yeast strains during chemostat cultures. Data were partly obtained from the references presented in Table 1 (some data were obtained from graphs). Data for *Kluyveromyces marxianus* ATCC 26548 are those obtained in the present study.

Considering these observations and the fact that the lipid content was higher under this condition, as compared with the other cultivations (Table 4), it seems that the higher availability of oxygen and/or the higher rate of CO_2 stripping has an effect on the physiology of *K. marxianus* ATCC 26548. Lipid biosynthesis, mainly that of sterols, is known to require oxygen (Rosenfeld & Beauvoit, 2003) and for some reason the higher availability of this compound in the chemostat at 0.1 h^{-1} and 2.5 v.v.m. than for the culture at the same dilution rate, but with 1 v.v.m. air sparging, results in a higher lipid content. However, oxygen can also have damaging effects for the cells, since it results in the formation of reactive oxygen species, and the fact that a greater amount of metabolites was observed when the culture at 0.1 h^{-1} was sparged with 2.5 v.v.m. than with 1 v.v.m. may be a consequence of some oxidative stress. Confirmation of this hypothesis would require further investigation. It is important to note that, despite the differences in oxygen supply, the dissolved oxygen concentration was always in the range of 100% saturation for both cultures at 0.1 h^{-1}. A further hypothesis is that a higher rate of CO_2 stripping, as a consequence of the higher aeration employed, has a negative effect on carboxylation reactions, e.g. that catalysed by pyruvate carboxylase. Besides a lower availability of CO_2 for the reaction itself, the expression of the pyruvate carboxylase gene might be decreased under the 2.5 v.v.m. condition, as it has been shown that transcription of the *PYC1* gene of *S. cerevisiae*, which codes for pyruvate carboxylase, increases with an increase in CO_2 availability (Aguilera *et al.*, 2005). If this is the case, there might be a lower formation of tricarboxylic acid (TCA) cycle intermediates, decreasing their availability as building blocks for biomass formation.

In the batch culture, a higher formation of metabolites was observed (around 20.5 h), as compared with the chemostat cultures, which is probably a consequence of the higher specific growth rate achieved under this condition. Again, pyruvate, 2-oxoglutarate and acetate were the predominant metabolites observed. As can be seen from Figs 1 and 2, all metabolites are rapidly consumed after glucose exhaustion.

Some, such as acetate, begin to be consumed even before glucose exhaustion, which indicates that glucose repression on the consumption of these other carbon sources is not as strong in this yeast as in *S. cerevisiae*, for which the metabolites formed during a batch cultivation on glucose only start being consumed some time after glucose exhaustion (Locher *et al.*, 1993).

Nevertheless, the total formation of metabolites during the batch cultures was still low compared with other yeasts. Considering this and the fact that some of the metabolites start being consumed even before glucose exhaustion, in addition to the results from a previous study with the same strain, in which no metabolite formation was detected (Bellaver *et al.*, 2004), it seems that metabolite formation and consumption remain either side of a critical boundary, eventually occurring simultaneously in this yeast, under these cultivation conditions. It should also be mentioned that, as discussed above, although the study performed by Bellaver *et al.* (2004) was carried out with the same strain, the maximum specific growth rate was lower in that study, which might also explain the lower formation of metabolites in that case.

According to van Urk *et al.* (1988) the different behaviour with regard to glucose assimilation (catabolism via respiration or fermentation to ethanol) in Crabtree-positive and Crabtree-negative yeasts is apparently not caused by differences in the glucose uptake rate or in the respiratory potential, as the glucose and the oxygen uptake rates were approximately the same for aerobic-respiring and aerobic-fermenting yeasts, when exposed to a glucose pulse applied to respiring cells. However, comparing CO_2 production and O_2 consumption in different yeasts (Table 1), we observe that the growth rate is a key factor for triggering aerobic fermentation in aerobic-respiring and aerobic-fermenting yeasts. The difference between these two classes of yeasts is that in the latter group the shift from respiratory to fermentative metabolism is reached at lower growth rates.

Glucose is known to trigger different global responses in yeast, as a function of its concentration (Meijer *et al.*, 1998; Yin *et al.*, 2003). However, as shown recently by Blank & Sauer (2004), glucose repression of the TCA cycle exhibits a different pattern and probably also uses different signals when compared with the paradigm glucose repression gene *SUC2* (Meijer *et al.*, 1998; Rolland *et al.*, 2002). In our studies, a concentration of 10 g glucose L^{-1} was not high enough to cause alcoholic fermentation in continuous cultures of *K. marxianus* at 0.1 h^{-1}. However, during the chemostat carried out at 0.25 h^{-1}, which was also a carbon-limited culture, ethanol was formed, although to a very low extent. Thus, there seems to be a compromise between the specific growth rate and the formation of ethanol, which is in accordance with the proposal of Blank & Sauer (2004) for repression of the TCA cycle.

Biomass composition

Given that the content of protein, carbohydrates, lipids, RNA, DNA and ash was always independently determined in the samples, and as their sum was always within a 99–101% range, the values presented in Table 4 can be considered to be accurate.

It is well established that there is proportionality between the specific growth rate of yeast cells and their cellular content of protein and RNA, i.e. the active machinery (Parada & Acevedo, 1983; Verduyn *et al.*, 1990, 1992). The protein content of *K. marxianus* cells during the batch cultures performed in this work is in close agreement with previously reported values for the same yeast species (Guiraud *et al.*, 1981; Lukondeh *et al.*, 2003; Table 4). These values also compare well with those reported for *S. cerevisiae* (Table 4).

The chemostat cultivation run at 0.5 h^{-1}, which was not carbon limited, as already discussed above, led to an unusually high protein content in the cells. Accordingly, the carbohydrate content was very low and RNA content was higher compared with the remaining chemostats. Data on the protein content of yeasts growing in chemostats at high dilution rates are not common in the literature, but Nissen *et al.* (1997) report a 60% protein content for *S. cerevisiae* cells growing at 0.4 h^{-1}, which is not much lower than the 71.9% value measured in the chemostat carried out with *K. marxianus* ATCC 26548 at 0.5 h^{-1} (Table 4).

As biomass composition values for *K. marxianus* yeasts are rare in the literature, we believe that the measurements reported in this work will be useful for future research on the physiology of these yeasts, e.g. for the application of metabolic flux analysis.

In summary, *K. marxianus* aligns with the so-called aerobic-respiring or Crabtree-negative yeasts in terms of respiration parameters and protein content. However, it has one of the highest growth rates among yeasts, and a high capacity of converting sugar into biomass, even when carbon is not the limiting nutrient. These results provide useful data regarding the future application of *K. marxianus* in processes aimed at the production of biomass-linked compounds, with high yields and productivities.

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