Research Article

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Protein enrichment of an *Opuntia ficus-indica* cladode hydrolysate by cultivation of *Candida utilis* and *Kluyveromyces marxianus*

Gabriel B Akanni, James C du Preez,* Laurinda Steyn and Stephanus G Kilian

Abstract

BACKGROUND: The cladodes of *Opuntia ficus-indica* (prickly pear cactus) have a low protein content; for use as a balanced feed, supplementation with other protein sources is therefore desirable. We investigated protein enrichment by cultivation of the yeasts *Candida utilis* and *Kluyveromyces marxianus* in an enzymatic hydrolysate of the cladode biomass.

RESULTS: Dilute acid pretreatment and enzymatic hydrolysis of sun-dried cladodes resulted in a hydrolysate containing (per litre) 45.5 g glucose, 6.3 g xylose, 9.1 g galactose, 10.8 g arabinose and 9.6 g fructose. Even though *K. marxianus* had a much higher growth rate and utilized L-arabinose and D-galactose more completely than *C. utilis*, its biomass yield coefficient was lower due to ethanol and ethyl acetate production despite aerobic cultivation. Yeast cultivation more than doubled the protein content of the hydrolysate, with an essential amino acid profile superior to sorghum and millet grains.

CONCLUSIONS: This *K. marxianus* strain was weakly Crabtree positive. Despite its low biomass yield, its performance compared well with *C. utilis*. This is the first report showing that the protein content and quality of *O. ficus-indica* cladode biomass could substantially be improved by yeast cultivation, including a comparative evaluation of *C. utilis* and *K. marxianus*.

Keywords: *Opuntia ficus-indica*; lignocellulose hydrolysate; *Kluyveromyces marxianus*; *Candida utilis*; single cell protein; yeast

INTRODUCTION

The prickly pear cactus, *Opuntia ficus-indica*, is well adapted for cultivation in semi-arid regions.[1] The cladodes, also known as cactus pads, of the spineless cultivars can be used as livestock feed, but because of their low crude protein content of about 40 g kg^{-1} they should be regarded as a cheap energy source rather than as a balanced fodder crop.[1–3] For instance, a ration for non-reproductive sheep should contain at least 70 g crude protein kg^{-1}; this necessitates the supplementation of cactus cladodes with some form of crude protein for use as a balanced animal feed.[1,4] Because of the high annual productivity of 10–40 tonnes (dry weight) of cladode biomass per hectare, the cladodes have potential as a lignocellulosic feedstock to produce a protein-enriched biomass product for use as an animal feed. Feedstocks such as molasses, cheese whey and hydrocarbons are the major carbon sources used for single-cell protein (SCP) production and, more recently, lignocellulosic feedstocks have received attention.[5,6]

Yeasts are suitable for SCP production because of their high protein and low nucleic acid content. *Candida utilis* has been widely used, but *Kluyveromyces marxianus* has potential for SCP production owing to its greater temperature tolerance (resulting in a bioprocess with lower cooling costs and which is less prone to microbial contamination) and ability to utilize a broader range of carbon substrates than *C. utilis*.[7–10] Whereas galactose and arabinose are poorly utilized by *C. utilis*, the ability of *K. marxianus* to utilize these sugars confers an advantage when grown on lignocellulosic hydrolysates. Furthermore, like *C. utilis*, *K. marxianus* has GRAS (Generally Recognized as Safe) status and is regarded as one of the most Crabtree-negative yeasts (i.e. does not produce ethanol under aerobic conditions), which is advantageous for SCP production since under aerobic conditions yeast biomass production from carbohydrates would be maximized because no carbon and energy are lost due to ethanol production.[8]

There are several contradictory reports on the composition of *O. ficus-indica* cladodes[2,3,11,12] that may be attributed to variable factors such as plant age, climate and geographical location. The dried and milled cladodes used in this study had a total carbohydrate content of 420 g kg^{-1} on a dry weight basis,[13] which was substantially less than other conventional lignocellulosic feedstocks such as sugar cane bagasse (666 g kg^{-1}) and corn stover (650 g kg^{-1}),[14,15] mainly due to the lower glucan and xylan content of the *O. ficus-indica* cladodes.

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We investigated the feasibility of enriching the protein content and quality of an enzymatic cladode hydrolysate of an *O. ficus-indica* cultivar by aerobic cultivation of *K. marxianus* and *C. utilis*, including a comparative evaluation of the performance of these two yeasts.

MATERIALS AND METHODS

Production of an *O. ficus-indica* cladode enzymatic hydrolysate

Fresh cladodes of *Opuntia ficus-indica* (cultivar 'Algerian') were harvested in May from a cactus plantation outside Bloemfontein, South Africa. The cladodes, having a high water content of about 880–950 g kg^{-1}, were cut into strips using a mechanical shredder, sun dried and subsequently hammer milled to a particle size of 1 mm. The resulting cladode meal, having a dry matter content of 963 ± 1 g kg^{-1}, was thoroughly mixed to ensure representative samples and stored in a sealed container at room temperature.

The pretreatment conditions had previously been optimized by means of a central composite response surface statistical design using dilute sulfuric acid and pretreatment time as variables at a fixed temperature of 120 °C.[13] A stock hydrolysate of the cladode meal was prepared in a 15 L stainless steel Biostat® C bioreactor (Sartorius Stedim Biotech, Göttingen, Germany) by mixing 2.5 kg dry meal in 8.4 L H_2SO_4 (15 g kg^{-1}) to obtain a solids loading of 300 g L^{-1}. The bioreactor was sealed and left overnight at 24 °C with slow stirring. Subsequenty, the contents were autoclaved *in situ* at 120 °C for 45 min, cooled to 50 °C and adjusted to pH 4.8 by automatic titration with 3 mol L^{-1} KOH or 1.5 mol L^{-1} H_2SO_4 with slow stirring at 300 rev min^{-1}. A mixture of commercial enzymes consisting of 15 FPU cellulase (Spezyme CP, Genencor, Leiden, Netherlands), 15 IU β-glucosidase (Novozym 188, Novozymes A/S, Bagsvaerd, Denmark) and 100 IU pectinase (Pectinex Ultra SP-L, Novozymes A/S) per gram of dry cladode meal was added directly to the slurry and maintained at 50 °C with slow stirring for 48 h to allow hydrolysis. Samples were withdrawn at intervals for sugar analysis and the final hydrolysate was collected in sterile 1 L bottles, which were stored at −20 °C.

Yeast strains

Kluyveromyces marxianus UOFS Y2791, isolated from a local agave plant (*Agave americana*), and *Candida utilis* NRRL Y1084 were obtained from the University of the Free State MIRCEN yeast culture collection. These cultures were maintained on GPY agar slants containing (per litre) 40 g glucose, 5 g peptone, 5 g yeast extract and 20 g agar, stored at 4 °C and subcultured every 2 months.

Inoculum preparation

Pre-cultures of *K. marxianus* and *C. utilis* were grown in a sterile medium containing (per litre) 5 g glucose, 0.25 g citric acid, 3 g yeast extract (Merck, Darmstadt, Germany), 5 g $(NH_4)_2SO_4$, 9.61 g KH_2PO_4, 0.76 g K_2HPO_4, 0.75 g $MgSO_4.7H_2O$, 0.05 g $CaCl_2.2H_2O$, 0.1 g NaCl and 1 mL of a trace elements stock solution, adjusted to pH 5.5 with 3 mol L^{-1} KOH prior to autoclaving. The trace elements stock solution contained (per litre) 3.5 g $FeSO_4.7H_2O$, 0.7 g $MnSO_4.7H_2O$, 1.1 g $ZnSO_4.7H_2O$, 0.1 g $CuSO_4.5H_2O$, 0.2 g $CoSO_4.6H_2O$, 0.13 g $Na_2MoO_4.2H_2O$, 0.2 g H_3BO_3, 0.04 g KI and 0.16 g $Al_2(SO_4)_3.18H_2O$.[16] A 500 mL Erlenmyer side-arm flask containing 50 mL of the above medium was inoculated with a loopful of cells from a 24 h agar slant of *K. marxianus* or *C. utilis* and incubated at 40 or 35 °C, respectively, on an orbital shaker at 200 rev min^{-1} to late exponential phase. These temperatures were the upper limit of the optimum temperature range obtained from temperature profiles previously determined. A 1 mL volume was subsequently transferred to a second shake flask containing the same medium, which was similarly incubated and immediately used to inoculate the bioreactor vessel.

Cultivation conditions

Bioreactor cultivation

Batch cultivations were carried out with *K. marxianus* and *C. utilis* at 40 and 35 °C, respectively, in a 1.6 L Biostat® B-plus stirred tank reactor fitted with an exhaust gas condenser cooled to 1 °C and using a 1 L culture volume. The pH was maintained at pH 5.0 by automatic titration with either 3 mol L^{-1} KOH or 1.5 mol L^{-1} H_2SO_4. The bioreactor vessel containing 950 mL medium was inoculated with 50 mL of the shake flask culture. The dissolved oxygen tension (DOT) was monitored with a polarographic pO_2 electrode (Mettler Toledo, Halstead, UK) and controlled above 30% of saturation by cascade control of the stirrer speed and the aeration rate in the range 1.0–3.0 L min^{-1}. Foaming was controlled by automatic addition of Dow Corning 1510 silicone antifoam (BDH Laboratory Supplies, Poole, UK).

Culture media

When used as carbon substrate, the frozen cladode hydrolysate was thawed and an 800 mL volume sterilized in the bioreactor vessel at 110 °C for 10 min, cooled to the desired cultivation temperature, supplemented with a 150 mL concentrate of a mineral salts solution (below) that had been sterilized by autoclaving at 121 °C for 20 min, which also served as diluent to obtain a more miscible slurry, and adjusted to pH 5.0. Mineral salts were added to all culture media at the following final concentrations (per liter): 0.5 g citric acid, 10 g NH_4Cl, 5 g KH_2PO_4, 0.5 g K_2HPO_4, 1 g $MgSO_4.7H_2O$, 0.1 g $CaCl_2.2H_2O$ and 2 mL of the trace elements stock solution described above. In the case of *K. marxianus* cultures, a filter-sterilized vitamin stock solution that contained (per liter) 0.025 g biotin, 0.5 g nicotinic acid, 0.5 g pyridoxine hydrochloride, 0.5 g thiamine hydrochloride (Sigma-Aldrich, Steinheim, Germany), 0.5 g calcium pantothenate (Merck), 0.1 g *p*-aminobenzoic acid (Hopkin & Williams Ltd, Chadwell Heath, UK) and 12.5 g *m*-inositol (BDH Laboratory Supplies)[17] was added to the sterile basal medium.

Two chemically defined media were also used, namely a simulated hydrolysate medium containing hexose and pentose sugars at similar concentrations as present in the enzymatic cladode hydrolysate, and a similar glucose-limited medium that contained glucose as the only sugar at a final concentration of 10 or 50 g L^{-1}. In each case 150 mL of the sterile concentrated sugar solution was added to 800 mL of the above mineral salts basal medium in the bioreactor vessel, which had been adjusted to pH 5.0 with 3 mol L^{-1} KOH and autoclaved at 121 °C for 20 min.

Culture samples were collected at regular intervals for analyses. All cultivations were carried out at least in duplicate and mean values are reported.

Analytical procedures

Cellulase and β-glucosidase activities were determined according to IUPAC guidelines,[18] and pectinase activity as described elsewhere.[19] Cell concentrations were monitored by measuring

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culture turbidity against a medium blank with a Photolab S6 spectrophotometer (WTW, Weilheim, Germany) at 690 nm. Dry cell weight was gravimetrically determined using duplicate 10 mL samples that were centrifuged, washed with distilled water and dried overnight at 105 °C. The CO_2 and O_2 content of the bioreactor exhaust gas was continuously monitored using an Uras 10E infrared and a Magnos 6G paramagnetic gas analyser (Hartman & Braun, Frankfurt, Germany) and the gas exchange rates were calculated by means of a nitrogen balance. During some of the *K. marxianus* cultivations, ethyl acetate in the exhaust gas was trapped with ORBO™ desorption tubes (Sigma-Aldrich, Deisenhofen, Germany) containing activated coconut charcoal as adsorbent and the ethyl acetate subsequently extracted from these tubes with dichloromethane (Sigma-Aldrich, Steinheim),[20] followed by analysis by gas chromatography (GC) as described below.

Samples collected for determining residual sugars and metabolic products were immediately cooled in ice before centrifugation at 10 600 × *g* and 4 °C using an Eppendorf 5430 R centrifuge (Eppendorf AG, Hamburg, Germany) and the supernatants filtered through a 0.45 µm acetate membrane filter (Pall, Port Washington, NY, USA) prior to analysis. Supernatants not immediately analysed were stored at −20 °C. Hydrolysate slurries were diluted to reduce the viscosity of the sample. The concentrations of glucose, xylose, galactose, arabinose and fructose were determined with a Waters high-performance liquid chromatography (HPLC) instrument (Waters Corp., Milford, MA, USA) using an Aminex HPX-87P column (Bio-Rad, Hercules, CA, USA) at 85 °C with MilliQ water at a flow rate of 0.4 mL min^{-1} as eluent, or with a Rezex RPM-Monosaccharide Pb^{+2} cation exchange column (Phenomenex, Torrance, CA, USA), the latter giving a better peak resolution. Ethanol, acetaldehyde and ethyl acetate were determined with a Shimadzu GC 2010 gas chromatograph (Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with a ZB wax column (Phenomenex) and a flame ionization detector with hydrogen as carrier gas at a linear velocity of 35 cm s^{-1}, using a 0.6 µL injection volume at a 50:1 split ratio. The oven temperature was 80 °C for 2.5 min, ramped at 25 °C min^{-1} to 180 °C with a 2 min isothermal period. The injector and detector temperatures were 150 and 300 °C, respectively. For determination of the ethyl acetate recovered from the bioreactor exhaust gas, an oven temperature of 40 °C for 5 min, ramped at 10 °C min^{-1} to 150 °C with a 5 min isothermal period was used, with a 1.0 µl injection volume at a 10:1 split ratio and injector and detector temperatures of 150 and 280 °C, respectively. A refractive index detector (Waters) was used for the determination of sugars.

The protein content of the cells was determined the by biuret method[21] using bovine serum albumin, fraction V (Sigma-Aldrich, St Louis, MO, USA) as protein standard. Amino acids were determined by transferring a washed biomass suspension equivalent to 0.5 mg dry mass into vacuum reaction hydrolysis tubes with norleucine as internal standard. Following liquid phase acid hydrolysis at 108–110 °C for 24 h to access the proteinogenic amino acids, these were derivatized by incubating with a 1:1 volumetric ratio of *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA) (Merck, Hohenbrunn, Germany) and *N*,*N*′-dimethylformamide (DMF) (Merck, Darmstadt, Germany) for 60 min at 60 °C.[22] The derivatized amino acids were analysed by gas chromatography–mass spectrometry (GC-MS) using a GC Trace Ultra instrument with a DSQ quadrupole mass spectrometer (Thermo Finnigan, San Jose, CA, USA).

Table 1. Sugar composition of *O. ficus-indica* cladode biomass after enzymatic hydrolysis

	Carbohydrates				
Parameter	Glucose	Xylose	Galactose	Arabinose	Fructose
Concentration (g L^{-1})	45.5	6.3	9.1	10.8	9.6
Sugar recovery (%)[a]	78.8	64.4	90.6	67.5	79.7

[a] Based on a cladode composition of 182 g glucose, 25 g xylose, 58 g galactose, 26 g arabinose and 38 g fructose per kilogram dry biomass.[13]

RESULTS

Composition of *O. ficus-indica* cladode hydrolysate

The sugar concentrations in the slurry resulting from acid pretreatment of the *O. ficus-indica* cladode dried meal were (per litre) 7.4 g glucose, 3.9 g xylose, 2.3 g galactose, 4.0 g arabinose and 5.0 g fructose. Subsequent hydrolysis with cellulase, *β*-glucosidase and pectinase released 78% of the theoretical sugar content based on previous analysis of this cladode biomass in our research group,[13] resulting in a hydrolysate with a carbohydrate composition as shown in Table 1 with a total sugar concentration of 81.3 g L^{-1}. These results served as a basis for formulating a chemically defined simulated hydrolysate culture medium.

Yeast cultivation in an enzymatic hydrolysate of cladode biomass

In the hydrolysate medium *K. marxianus* and *C. utilis* preferentially utilized glucose followed by fructose uptake (Fig. 1). After 50 h of cultivation *K. marxianus* had consumed 97% and *C. utilis* only 74% of the total sugars. *K. marxianus* utilized galactose, arabinose and xylose mainly after exhaustion of the glucose and, with the exception of arabinose, the sugars were almost completely consumed within 30 h. *Candida utilis* consumed glucose, fructose and xylose within 35 h, but at the end of the cultivation only 32% of the galactose and 11% of the arabinose had been consumed (Fig. 1). The volumetric rate of total sugar uptake by *K. marxianus* was 2.63 g L^{-1} h^{-1}, which was substantially faster than *C. utilis* (Table 2). *Candida utilis* produced small amounts of glycerol and acetic acid, whereas *K. marxianus* produced up to 2.9 g ethanol L^{-1} as well as low concentrations of acetic acid and ethyl acetate, despite the aerobic culture conditions (Table 2).

The cellular protein content of *C. utilis*, grown in a chemicaly defined medium and determined by the biuret method, was 530 g kg^{-1} on a dry weight basis, whereas that of *K. marxianus* was 443 g kg^{-1}. The yeast dry biomass concentration in the cladode hydrolysate could not directly be determined owing to the viscosity and presence of particulate matter. Therefore the yeast biomass was indirectly estimated from biuret protein assays of the hydrolysate culture broth, correcting for the initial content of plant protein. These estimates indicated that *K. marxianus* produced a dry biomass concentration of 11.1 g L^{-1}, whereas the *C. utilis* biomass was slightly higher at 12.2 g L^{-1} (Fig. 1). The biomass yields of both yeasts were lower than expected, especially that of *K. marxianus* (Table 2). Cultivation of *K. marxianus* and *C. utilis* increased the total protein content of the hydrolysate, on a dry weight basis, from an initial value of 80 g kg^{-1} to 180 and 220 g kg^{-1}, respectively (Table 2).

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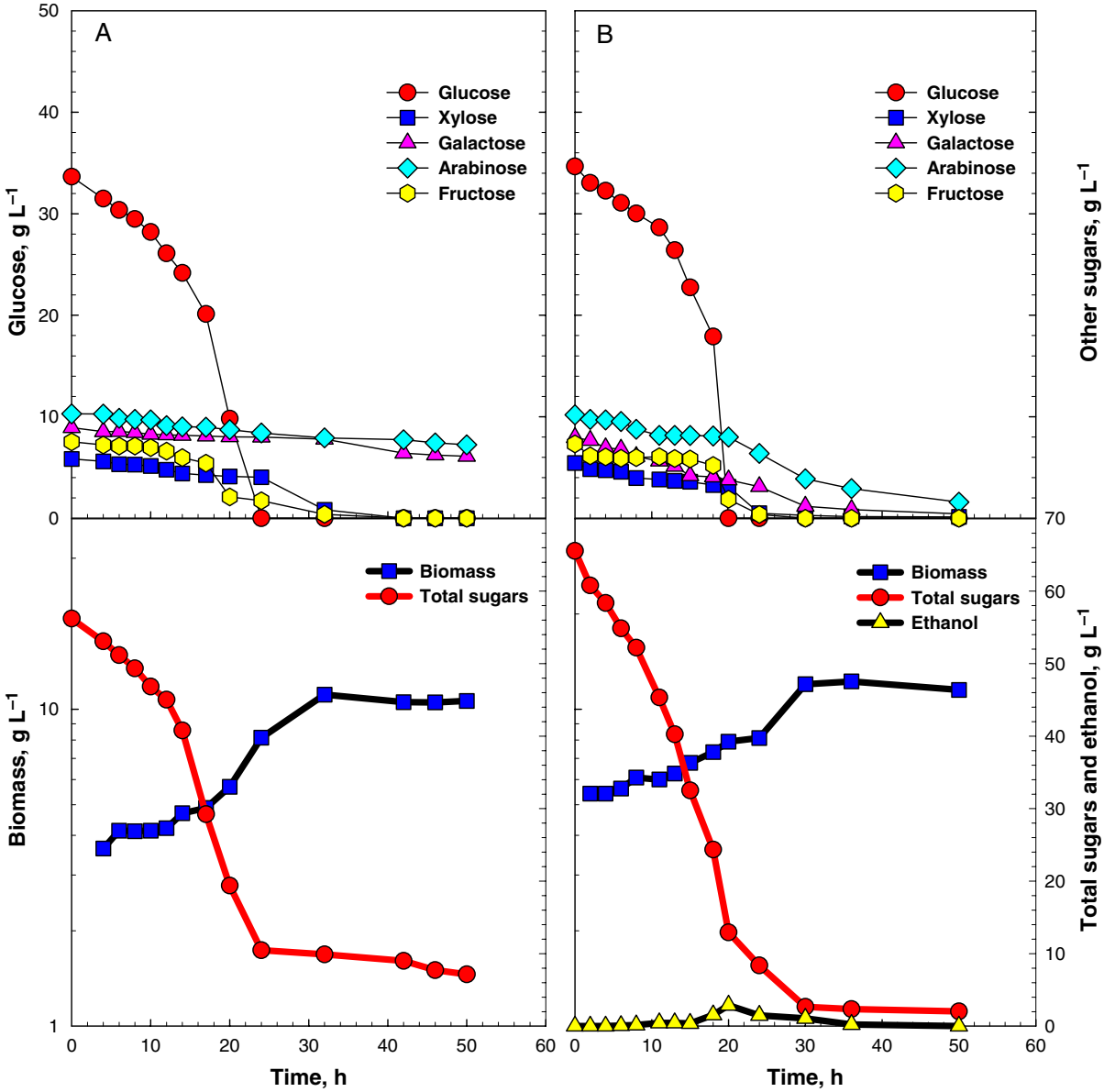

Figure 1. Cultivation profiles of *C. utilis* (A) and *K. marxianus* (B) with the DOT controlled above 30% of saturation in an enzymatic hydrolysate of *O. ficus-indica* cladode biomass supplemented with mineral salts.

Yeast cultivation in a simulated hydrolysate medium

To serve as a benchmark for the performance of yeasts in the cladode hydrolysate and to investigate the low biomass yield found with *K. marxianus*, experiments were conducted using a chemically defined medium containing a sugar mixture simulating the composition of the cladode hydrolysate to facilitate carbon and degree of reduction balances, using a biomass composition of $CH_{1.8}O_{0.5}N_{0.2}$. In this medium *K. marxianus* had a high maximum specific growth rate (μ_{max}) of 0.71 h^{-1}, whereas *C. utilis* had a μ_{max} value of 0.40 h^{-1} and a biomass yield coefficient of 0.42. By contrast, the biomass yield coefficient of *K. marxianus* remained low at 0.24 (Table 3). The sugar utilization profiles were similar to those in the actual cladode hydrolysate medium (Fig. 2). Although arabinose utilization by *K. marxianus* appeared slightly slower than in the actual hydrolysate, the volumetric rate of total sugar uptake by both yeasts was about 1.5-fold higher than in the hydrolysate. After 30 h of cultivation, *K. marxianus* had utilized about 88% of the total sugars and *C. utilis* 79%. *Kluyveromyces marxianus* again produced up to 6.4 g ethanol L^{-1} as well as ethyl acetate, acetaldehyde and acetic acid, whereas *C. utilis* produced glycerol and acetic acid (Fig. 2 and Table 3). In this medium both yeasts produced a substantially higher acetic acid concentration than in the actual hydrolysate. The carbon and degree of reduction balances for *C. utilis* cultivation closed to 97% and 94%, respectively, with a respiratory quotient (RQ) of 1.12. However, in the case of *K. marxianus* the carbon and degree of reduction balances closed to only 65% and 72%, respectively, and the RQ of 1.86 indicated a respiro-fermentative metabolism.

Yeast cultivation in a glucose-limited medium

To further investigate the low biomass yields of *K. marxianus* and the failure of the balances of its cultivation to close, the yeasts were grown aerobically in a chemically defined glucose-limited medium. Using an initial glucose concentration of 10 g L^{-1},

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Table 2. Growth parameters of *C. utilis* and *K. marxianus* in an enzymatic hydrolysate of *O. ficus-indica* cladode biomass supplemented with mineral salts (mean values are shown)

Parameter	*C. utilis*	*K. marxianus*
Residual sugars (g L^{-1})	21.75	2.91
$Y_{x/s}$ (g g^{-1})	0.25	0.19
$Y_{p/s}$ (g g^{-1})	0	0.07
Q_s (g sugars L^{-1} h^{-1})	1.91	2.63
Ethanol (g L^{-1})	ND	2.90
Ethyl acetate (g L^{-1})	ND	0.57
Acetic acid (g L^{-1})	0.35	0.45
Glycerol (g L^{-1})	0.02	0
Initial protein concentration (g L^{-1})	0.50	0.50
Final protein concentration (g L^{-1})	6.0	5.40
Initial protein content (g kg^{-1})[a]	80	80
Final protein content (g kg^{-1})[a]	220	180
Initial dry plant biomass concentration (g L^{-1})	24.11	24.11
Final total dry biomass concentration (g L^{-1})[b]	34.58	34.13

$Y_{x/s}$, biomass yield coefficient on total sugars assimilated (estimated from biuret protein assays); $Y_{p/s}$, ethanol yield coefficient on total sugars assimilated; Q_s, maximum volumetric rate of total sugars uptake, calculated from the slope of the curve of total sugar concentration *versus* time; ND, not detected.
[a] Expressed in terms of total dry weight of washed solids.
[b] Total of plant and yeast biomass solids.

Table 3. Growth parameters of *C. utilis* and *K. marxianus* in a chemically defined medium containing a sugar mixture simulating an enzymatic hydrolysate of cladode biomass (mean values are shown)

Parameter	*C. utilis*	*K. marxianus*
Residual sugars (g L^{-1})	17.21	9.60
μ_{max} (h^{-1})	0.40	0.71
$Y_{x/s}$ (g g^{-1})	0.46	0.24
$Y_{p/s}$ (g g^{-1})	0	0.12
Q_s (g sugars L^{-1} h^{-1})	2.59	4.05
Ethanol (g L^{-1})	ND	6.44
Ethyl acetate (g L^{-1})	ND	0.34
Acetaldehyde (g L^{-1})	ND	0.25
Acetic acid (g L^{-1})	1.72	2.54
Glycerol (g L^{-1})	0.01	0
Final protein concentration (g L^{-1})	11	6.2

μ_{max}, maximum specific growth rate; $Y_{x/s}$, biomass yield coefficient on total sugars assimilated; $Y_{p/s}$, ethanol yield coefficient on total sugars assimilated; Q_s, maximum volumetric rate of total sugars uptake, calculated from the slope of the curve of product concentration *versus* time; ND, not detected.

K. marxianus and *C. utilis* NRRL Y-1084 exhibited μ_{max} values of 0.78 and 0.42 h^{-1}, respectively, with corresponding biomass yield coefficients of 0.45 and 0.50 (Fig. 3). *Kluyveromyces marxianus* produced a minimal amount of ethanol (0.23 g L^{-1}) under these conditions. However, with an initial glucose concentration of 50 g L^{-1}, the biomass yield coefficient of *K. marxianus* decreased to 0.15, while ethanol production increased to 8.4 g L^{-1} with the accumulation of lower concentrations of glycerol (1.3 g L^{-1}), acetic acid (0.75 g L^{-1}), acetaldehyde (0.11 g L^{-1}) and ethyl acetate (0.25 g L^{-1}). By contrast, the biomass yield of *C. utilis* remained close to the theoretical maximum, irrespective of the glucose concentration, with the production of less than 0.05 g L^{-1} glycerol and acetic acid but no ethanol (Fig. 3). Thus, although *K. marxianus* is regarded as strongly Crabtree negative, the above observations suggested a shift from a respiratory to a respiro-fermentative metabolism at the higher sugar concentration, as indicated by aerobic ethanol production and a concomitant increase in the RQ to 1.98.

Whereas the carbon and degree of reduction balances, based on gas exchange values and metabolites in the culture broth, of *K. marxianus* grown in a medium containing 10 g glucose L^{-1} closed to within 96% and 98%, respectively, in the medium containing 50 g glucose L^{-1} the balances failed to close by a large margin. Increasing the vitamin supplementation twofold or supplementing the medium with yeast extract at 3 g L^{-1} failed to improve the biomass yield of *K. marxianus*. However, in cultivations with the DOT maintained at above 50% of saturation, its μ_{max} increased slightly to 0.80 h^{-1}, with a substantial increase in the biomass yield coefficient of up to 0.31 and ethanol production decreasing to below 2.13 g L^{-1}, but still with a poor recovery of carbon and reducing equivalents in the balances (data not shown).

It was subsequently discovered that the failure of the balances to close was due to the stripping of highly volatile ethyl acetate from the culture broth by aeration, despite an exhaust gas condenser cooled to 1 °C on the culture vessel. The amount of ethyl acetate recovered by fitting an activated charcoal column on the exhaust gas condenser corresponded to a concentration of 12.05 g L^{-1} in the culture broth, while the actual ethyl acetate in the culture broth amounted to only 0.51 g L^{-1}. By including both these amounts, the carbon and degree of reduction balances resulted in recoveries of 108% and 103%, respectively.

Amino acid composition of yeast-enriched cladode hydrolysate

Table 4 shows the amino acid profiles – except for tryptophan, asparagine and glutamine, which were destroyed during acid hydrolysis, of the cladode hydrolysate and the biomass product resulting from the cultivation of *C. utilis* and *K. marxianus* in the hydrolysate. Yeast cultivation effected a two- to threefold increase in the content of almost all the amino acids, with the lysine content increasing about fivefold and the total amino acid content increasing almost 2.9-fold.

The essential amino acid profile of the yeast-enriched hydrolysate protein compared quite favourably with the FAO/WHO scoring pattern,[23] with the exception of the sulfur-containing amino acids and lysine (Table 5). Even though yeast cultivation greatly increased the lysine content of the cladode hydrolysate (Table 4), the ratio of the lysine content of the biomass protein to the value in the FAO/WHO scoring pattern was only 0.49 to 0.5 (Table 5). Owing to the low content of methionine and cystine, the chemical score of the protein of the biomass product obtained by cultivation of *C. utilis* and *K. marxianus* was 49% and 47%, respectively.

DISCUSSION

The main drawback in using the cladode biomass as feedstock for yeast cultivation to enhance its protein content and amino acid profile was the very high water content of the cladodes. This problem was addressed by sun-drying and milling the fresh cladodes, giving a more concentrated feedstock as well as facilitating easier storage and handling. The total sugar content of the cladode

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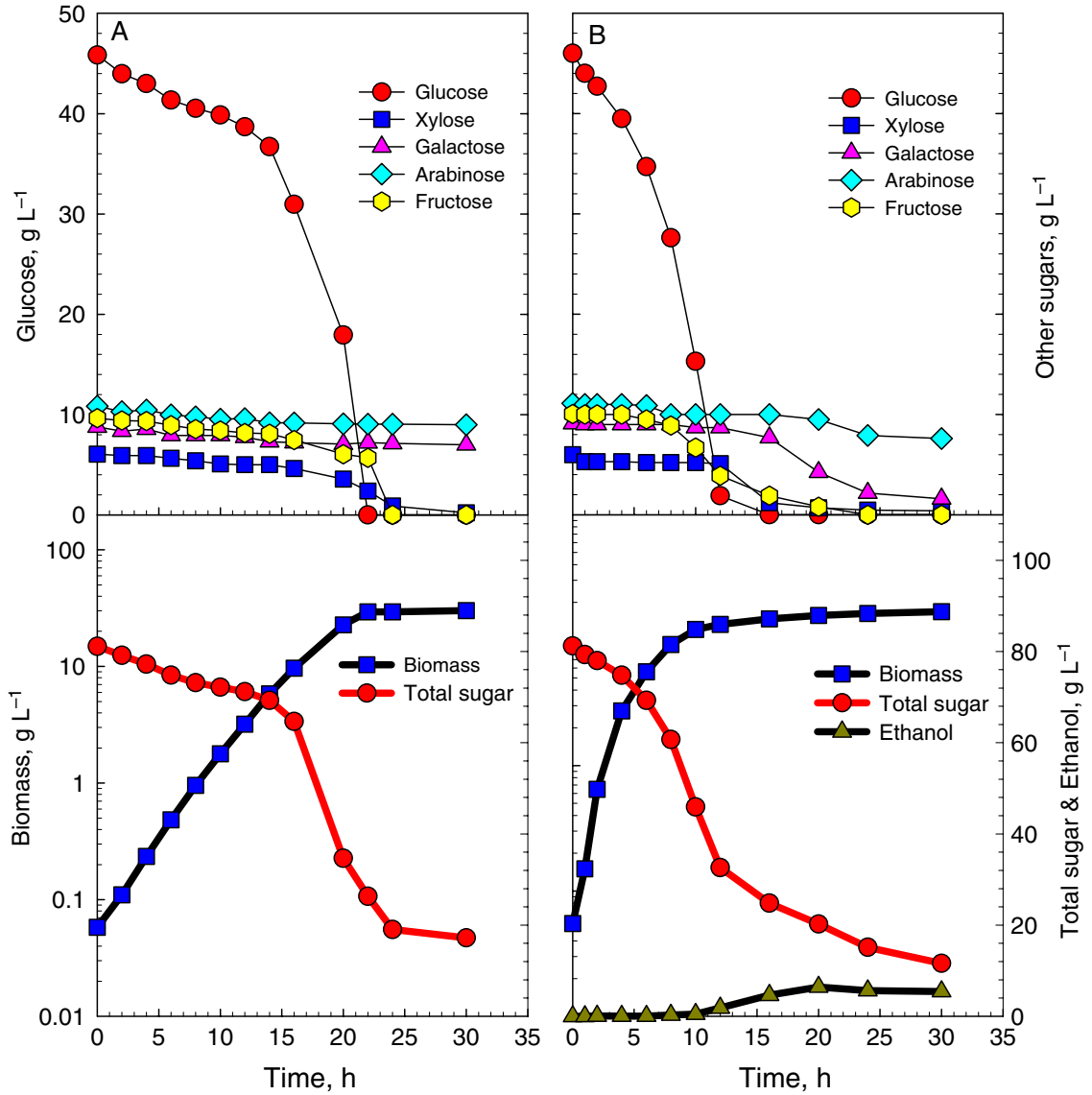

Figure 2. Cultivation profiles of *C. utilis* (A) and *K. marxianus* (B) with the DOT controlled above 30% of saturation in a chemically defined medium containing a sugar mixture simulating an enzymatic hydrolysate of cladode biomass.

hydrolysate, 81.3 g L^{-1}, was less than found in conventional lignocellulosic hydrolysates,[14,24,25] mainly due to the lower glucan and xylan content of the cladodes.

Although regarded as strongly Crabtree negative,[8] the biomass yield of *K. marxianus* was substantially less than that of *C. utilis* due to the formation of by-products such as ethanol, despite fully aerobic cultivation conditions. This indicated that this strain of *K. marxianus* was in fact weakly Crabtree positive, exhibiting a degree of respiro-fermentative metabolism. There are differing reports regarding the Crabtree characteristic of *K. marxianus* and these variations were apparently strain dependent.[26–28] For example, it was reported that *K. marxianus* DSM 5420 produced 10 g ethanol L^{-1} from 120 g lactose L^{-1} under strict aerobic conditions.[10] A shift to ethanol production could be a consequence of an imbalance between glycolysis and the respiratory chain, or to an oxygen, thiamine or iron limitation.[8,29,30] A respiro-fermentative metabolism was observed in a continuous culture of a *K. lactis* strain when the dilution rate was increased to above 0.4 h^{-1}; in this case ethanol production was attributed to a nicotinamide limitation.[31] All *K. marxianus* strains have an absolute nicotinamide requirement.[10,32] However, we found that increasing the vitamin supplementation and even adding yeast extract at 3 g L^{-1} had little effect on aerobic ethanol production by our strain of *K. marxianus* (data not shown), making it unlikely that ethanol production in our cultures was triggered by a nutrient or growth factor limitation.

Some *K. marxianus* strains have a strong tendency to produce pyruvate and acetate when exposed to excess sugar.[33] We detected also substantial production of ethyl acetate by *K. marxianus*, as has previously been found with some strains grown under an iron limitation.[29] In our cultivations ethyl acetate production was unexpected, since the iron content of the medium was in excess and no trace element limitation was likely. The proposed mechanism of ethyl acetate synthesis by *K. marxianus* is either from ethanol and acetyl-CoA catalyzed by an alcohol acetyltransferase, or from ethanol and acetate catalyzed by an esterase with oxygen as an obligatory requirement for either of these reactions.[29,34] Ethyl acetate is more volatile than ethanol and rapid stripping of

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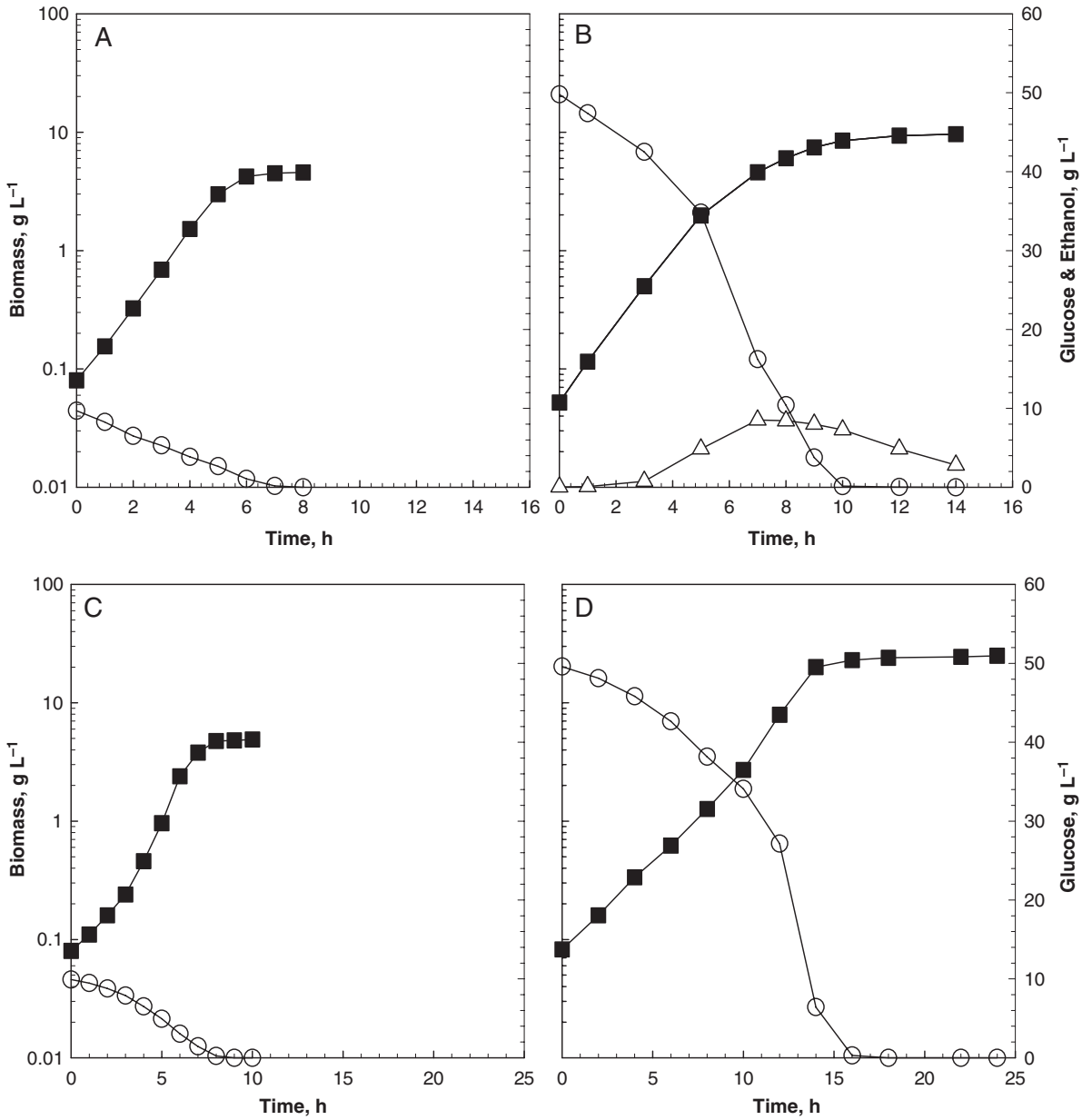

Figure 3. Effect of glucose concentration on the cultivation profiles of *K. marxianus* (A, B) and *C. utilis* (C, D) in a chemically defined medium maintained at a DOT of above 30% of saturation and containing a glucose concentration of 10 (A, C) or 50 g L^{-1} (B, D). Symbols: biomass (■); glucose (○); ethanol (△).

this ester from the culture broth occurs during aerobic cultivation. This, together with ethanol production, accounted for the low biomass yield obtained with *K. marxianus* and the failure of the carbon and degree of reduction balances to close until we could account for the ethyl acetate lost in the bioreactor exhaust gas. Subsequently, the recovery of reducing equivalents and carbon slightly exceeded 100%. This could have been due to experimental error and/or the 'standard' elemental biomass composition used in these calculations differing from the actual composition of *K. marxianus*.

The protein content of *C. utilis*, 530 g kg^{-1} (dry weight), as determined by the biuret assay, was comparable to values previously reported for this yeast,[35,36] whereas the protein content of *K. marxianus* was only 443 g kg^{-1}. However, despite its lower protein content, cultivation of *K. marxianus* in the cladode hydrolysate increased the total protein concentration to 5.4 g L^{-1}, which was slightly less than the 6 g L^{-1} obtained with *C. utilis*, probably because *K. marxianus* was capable of utilizing more of the hydrolysate sugars. The relatively high content of lysine and threonine in yeast biomass renders the latter well suited for supplementation of plant-based feeds.[10] The lysine content of *C. utilis* and *K. marxianus* (grown in a simulated cladode hydrolysate) was 26.8 and 32.1 g kg^{-1} dry cell mass, respectively (data not shown). Although their cultivation in the cladode hydrolysate increased the lysine content about fivefold (Table 4), the lysine content of the final biomass product amounted to only 49% of the value of the FAO/WHO scoring pattern (Table 5). Similarly, the initial content of methionine plus cystine in the cladode hydrolysate was increased more than twofold, but these sulfur-containing amino acids remained limiting, giving the protein of the biomass product a chemical score of 47% (Table 5). Nevertheless, the profile of essential amino acids in the protein obtained by enrichment

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Table 4. Amino acid profiles of *O. ficus-indica* cladode hydrolysate and of the biomass product (g 100 g^{-1} dry weight) obtained by cultivation of *C. utilis* and *K. marxianus*. The mean values of triplicate determinations and the standard deviation of the mean are shown.

Amino acids	Hydrolysate	Hydrolysate with *C. utilis*	Hydrolysate with *K. marxianus*
Isoleucine	0.37 ± 0.01	0.89 ± 0.00	0.91 ± 0.02
Leucine	0.59 ± 0.01	1.28 ± 0.01	1.32 ± 0.00
Lysine	0.15 ± 0.00	0.82 ± 0.05	0.72 ± 0.00
Methionine	0.07 ± 0.00	0.32 ± 0.04	0.18 ± 0.00
Phenylalanine	0.30 ± 0.01	0.69 ± 0.04	0.71 ± 0.04
Threonine	0.38 ± 0.03	1.27 ± 0.00	2.25 ± 0.02
Valine	0.49 ± 0.02	1.15 ± 0.01	1.19 ± 0.00
Cystine	0.11 ± 0.01	0.20 ± 0.00	0.26 ± 0.00
Arginine	0.13 ± 0.00	0.16 ± 0.00	0.21 ± 0.00
Histidine	0.03 ± 0.00	0.32 ± 0.00	0.31 ± 0.04
Alanine	0.50 ± 0.02	1.08 ± 0.00	1.26 ± 0.00
Aspartic acid	0.70 ± 0.01	1.96 ± 0.01	1.94 ± 0.02
Glutamic acid	0.63 ± 0.00	1.58 ± 0.00	1.72 ± 0.00
Glycine	0.36 ± 0.01	0.50 ± 0.02	0.80 ± 0.00
Proline	0.29 ± 0.02	0.67 ± 0.00	0.74 ± 0.04
Serine	0.37 ± 0.01	1.11 ± 0.01	1.20 ± 0.02
Tyrosine	0.25 ± 0.00	0.77 ± 0.00	0.71 ± 0.03
Total amino acids	5.72	16.48	16.41

Table 5. Essential amino acid composition (mg g^{-1} protein) of the protein in the biomass product obtained by cultivation of *C. utilis* and *K. marxianus*

Amino acid	FAO/WHO scoring pattern[a] (mg g^{-1})	Hydrolysate protein with *C. utilis* (mg g^{-1})	Ratio[b]	Hydrolysate protein with *K. marxianus* mg g^{-1}	Ratio†
Isoleucine	40	29.7	0.74	33.7	0.84
Leucine	70	42.7	0.61	48.9	0.70
Lysine	55	27.3	0.50	26.7	0.49
Methionine + cystine	35	17.3	0.49	16.3	0.47
Phenylalanine + tyrosine	60	48.6	0.81	52.6	0.88
Threonine	40	42.3	1.06	33.3	0.83
Tryptophan	10	nd	nd	nd	nd
Valine	50	38.3	0.77	44.1	0.88

[a] FAO/WHO.[23]
[b] Ratio of the amino acid content of the biomass protein to the essential amino acid in the FAO/WHO scoring pattern.
nd, not determined.

of the cladode hydrolysate through yeast cultivation compared favourably with cereals such as sorghum grains (chemical score of 37%) and millet grains (chemical score ranging from 31% to 63%).[37]

In addition to the chemical score, the essential amino acid index (EAAI) may be calculated from the essential amino acid profile to predict the nutritional value of a protein.[38] Again using the FAO/WHO scoring pattern but without tryptophan, which we did not determine, the EAAI values for the biomass product obtained using *C. utilis* and *K. marxianus*, respectively, were 68.8% and 70.1%. These compared favourably with the EAAI values reported for wheat flour (74–84%) and peanut flour (73–86%).[39]

CONCLUSIONS

The cladodes of *O. ficus-indica*, a plant well adapted for cultivation in semi-arid regions with a high annual productivity, have potential as a lignocellulosic feedstock for SCP production by yeast cultivation. The very high water content of the cladodes and the high viscosity of the enzymatic hydrolysate of the cladode biomass are constraints as to its use, however.

Using *K. marxianus* in a bioprocess has the advantages of a higher growth rate, a higher temperature tolerance and utilisation of a broader carbohydrate substrate range than possible with most other yeasts. These advantages were offset, however, by the unexpected low biomass yield of *K. marxianus* under certain cultivation conditions due to the production of ethanol and ethyl acetate. Aerobic ethanol production in yeasts is a complex phenomenon that has not yet been thoroughly elucidated in *K. marxianus*; the physiology of this as well as of ethyl acetate production warrant further investigation. It may be worthwhile to screen for strains with minimal production of these by-products to maximise biomass production, considering the favourable results obtained with this strain of *K. marxianus*.

Cultivation of *C. utilis* and *K. marxianus* in the cladode hydrolysate improved the total protein content (i.e. plant plus yeast protein) of the biomass product from an initial value of 80 g kg^{-1} to 220 and 180 g kg^{-1} (dry weight), respectively, which had an amino acid profile superior to that of cereals, with the exception of the sulfur-containing amino acids. Although the lysine content was increased by about fivefold, it was the second limiting essential amino acid. Animal feeding trials are required to ascertain the nutritional value of the biomass product, also taking into account digestibility.

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REFERENCES

1 De Kock GC, Drought resistant fodder shrub crops in South Africa, in *Browse in Africa: The Current State of Knowledge*, ed. by Le Houérou HN. International Livestock Centre for Africa, Addis Ababa, Ethiopia, pp. 399–408 (1980).
2 Malainine ME, Dufresne A, Dupeyre D, Mahrouz M, Vuong R and Vignon MR, Structure and morphology of cladodes and spines of *Opuntia ficus-indica*: cellulose extraction and characterisation. *Carbohydr Polym* **51**:77–83 (2003).
3 Stintzing FC and Carle R, Cactus stems (*Opuntia* spp.): a review on their chemistry, technology, and uses. *Mol Nutr Food Res* **49**:175–194 (2005).
4 Hamdi M, Prickly pear cladodes and fruits as a potential raw material for the bioindustries. *Bioprocess Eng* **17**:387–391 (1997).
5 Chanda S and Chakrabarti S, Plant origin liquid waste: a resource for single cell protein production by yeast. *Bioresour Technol* **57**:51–54 (1996).
6 Rajoka MI, Khan SH, Jabbar MA, Awan MS and Hashmi AS, Kinetics of batch single cell protein production from rice polishings with *Candida utilis* in continuously aerated tank reactors. *Bioresour Technol* **97**:1934–1941 (2006).
7 Fonseca GG, Gombert AK, Heinzle E and Wittmann C, Physiology of the yeast *Kluyveromyces marxianus* during batch and chemostat cultures with glucose as the sole carbon source. *FEMS Yeast Res* **7**:422–435 (2007).
8 Fonseca GG, Heinzle E, Wittmann C and Gombert AK, The yeast *Kluyveromyces marxianus* and its biotechnological potential. *Appl Microbiol Biotechnol* **79**:339–354 (2008).

9 Rouwenhorst RJ, Visser LE, van der Baan AA, Scheffers WA and van Dijken JP, Production, distribution, and kinetic properties of inulinase in continuous cultures of *Kluyveromyces marxianus* CBS 6556. *Appl Environ Microbiol* **54**:1131–1137 (1988).
10 Schultz N, Chang L, Hauck A, Reuss M and Syldatk C, Microbial production of single-cell protein from deproteinized whey concentrates. *Appl Microbiol Biotechnol* **69**:515–520 (2006).
11 Retamal N, Duran JM and Fernandez J, Ethanol production by fermentation of fruits and cladodes of prickly pear cactus (*Opuntia ficus-indica* (L.) Miller). *J Sci Food Agric* **40**:213–218 (1987).
12 Rodriguez-Felix A and Cantwell M, Developmental changes in composition and quality of prickly pear cactus cladodes (nopalitos). *Plant Food Hum Nutr* **38**:83–93 (1988).
13 Kuloyo OO, Ethanol production by yeast fermentation of an *Opuntia ficus-indica* biomass hydrolysate. MSc dissertation, University of the Free State, Bloemfontein, South Africa (2012).
14 Neureiter M, Danner H, Thomasser C, Saidi B and Braun R, Dilute-acid hydrolysis of sugarcane bagasse at varying conditions. *Appl Biochem Biotechnol* **98–100**:49–58 (2002).
15 Schell DJ, Riley CJ, Dowe N, Farmer J, Ibsen KN, Ruth MF *et al.*, A bioethanol process development unit: initial operating experiences and results with a corn fiber feedstock. *Bioresour Technol* **91**:179–188 (2004).
16 du Preez JC and van der Walt JP, Fermentation of D-xylose to ethanol by a strain of *Candida shehatae*. *Biotechnol Lett* **5**:357–362 (1983).
17 Schulze U, Anaerobic physiology of *Saccharomyces cerevisiae*. PhD thesis, Technical University of Denmark (1995).
18 Ghose TK, Measurement of cellulase activities. *Pure Appl Chem* **59**:257–268 (1987).
19 Phutela U, Dhuna V, Sandhu S and Chadha BS, Pectinase and polygalacturonase production by a thermophilic *Aspergillus fumigatus* isolated from decomposting orange peels. *Braz J Microbiol* **36**:63–69 (2005).
20 NIOSH (National Institute for Occupational Safety and Health), Ethyl acetate, Method 1457. *Manual of Analytical Methods*, 4th edn, Issue 1 (1994). [Online]. Available: http://www.cdc.gov/niosh/docs/2003-154/pdfs/1457.pdf [17 February 2014].
21 Herbert D, Phipps PJ and Strange RE, Chemical analysis of micobial cells, in *Methods in Microbiology*, vol. 5B, ed. by Norris JR and Ribbons DW. Academic press, New York, pp. 209–344 (1971).
22 Christensen B and Nielsen J, Isotopomer analysis using GC-MS. *Metab Eng* **1**:282–290 (1999).
23 Food and Agriculture Organization/World Health Organization, *WHO Technical Report Series No. 522*, FAO Nutrition Meetings Report Series No. 52. FAO/WHO, Geneva (1973). [Online]. Available: http://whqlibdoc.who.int/trs/WHO_TRS_522.pdf [1 April 2014].
24 García-Aparicio MP, Oliva JM, Manzanares P, Ballestros M, Ballesteros I, González A *et al.*, Second-generation ethanol production from steam exploded barley straw by *Kluyveromyces marxianus* CECT 10875. *Fuel* **90**:1624–1630 (2011).
25 Ruth MF and Thomas SR, The effect of corn stover composition on ethanol process economics, in *25th Symposium on Biotechnology for Fuels and Chemicals* (2003). [Online]. Available: http://www1.eere.energy.gov/biomass/pdfs/34040.pdf [6 March 2014].
26 Lane MM, Burke N, Karreman R, Wolfe KH, O'Byrne CP and Morrissey JP, Physiological and metabolic diversity in the yeast *Kluyveromyces marxianus*. *Antonie van Leeuwenhoek* **100**:507–519 (2011).
27 Lane MM and Morrissey JP, *Kluyveromyces marxianus*: a yeast emerging from its sister's shadow. *Fungal Biol Rev* **24**:17–26 (2010).
28 Rocha SN, Abrahão-Neto J and Gombert AK, Physiological diversity within the *Kluyveromyces marxianus* species. *Antonie van Leeuwenhoek* **100**:619–630 (2011).
29 Löser C, Urit T, Nehl F and Bley T, Screening of *Kluyveromyces* strains for the production of ethyl acetate: design and evaluation of a cultivation system. *Eng Life Sci* **11**:1–13 (2011).
30 Silva-Santisteban BO, Converti A and Filho FM, Effects of carbon and nitrogen sources and oxygenation on the production of inulinase by *Kluyveromyces marxianus*. *Appl Biochem Biotechnol* **152**:249–261 (2009).
31 Kiers J, Zeeman AM, Luttik M, Thiele C, Castrillo JI, Steensma HY *et al.*, Regulation of alcoholic fermentation in batch and chemostat cultures of *Kluyveromyces lactis* CBS 2359. *Yeast* **14**:459–469 (1998).
32 Kurtzman CP and Fell JW, *The Yeasts: A Taxonomic Study*. Elsevier Science, Amsterdam, pp. 154–165 (1998).
33 Hensing M, Vrouwenvelder H, Hellinga C, Baartmans R and van Dijken H, Production of extracellular inulinase in high-cell-density fed-batch cultures of *Kluyveromyces marxianus*. *Appl Microbiol Biotechnol* **42**:516–521 (1994).
34 Kallel-Mhiri H and Miclo A, Mechanism of ethyl acetate synthesis by *Kluyveromyces fragilis*. *FEMS Microbiol Lett* **111**:207–212 (1993).
35 Prior BA, Continuous growth kinetics of *Candida utilis* in pineapple cannery effluent. *Biotechnol Bioeng* **26**:748–752 (1984).
36 Tuntpatchalern S and Vananuvat P, Production of yeast protein by *Candida utilis* from pineapple juice II. Batch culture study. *J Sci Soc Thailand* **4**:36–44 (1987).
37 Food and Agriculture Organization, *Sorghum and millets in human nutrition*. FAO Food and Nutrition Series, No. 27 (1995). [Online]. Available: http://www.fao.org/docrep/T0818E/T0818E00.htm [1 April 2014].
38 Mitchell HH, The wastage of nutrients in metabolism: Proteins and amino acids, in *Comparative Nutrition of Man and Domestic Animals*. Academic Press, New York, pp. 567–659 (1964).
39 Seligson FH and Mackey LN, Variable predictions of protein quality by chemical score due to amino acid analysis and reference pattern. *J Nutr* **114**:682–691 (1984).

