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Thermotolerant single cell protein production by *Kluyveromyces marxianus* var. *marxianus*

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SUMMARY

Amino acid analyses were undertaken on single cell protein (SCP) produced by thermotolerant strains of *Kluyveromyces marxianus* var. *marxianus* grown on sugar cane molasses at 40°C. The maximum conversion of available sugars to biomass at 45°C was only 10.8% (g dry wt. · g^{-1} total sugars). The amino acid composition of the SCP did not differ markedly from that reported for other yeast species.

INTRODUCTION

Traditionally, yeasts such as *Candida utilis* and *Saccharomyces cerevisiae* have been used in the production of single cell protein (SCP) from molasses [5,15]. *Kluyveromyces* strains have been studied for SCP production from lactose [5,15], inulin [3,7], and beet sugar mill wastewater [13]. The high growth rate and comparatively high protein yields of *K. marxianus* from molasses-based substrates [3,6,12] and the tolerance of this species to temperatures greater than 40°C may make it suitable for SCP production in tropical areas.

Ambient temperatures in tropical sugar-cane-growing areas necessitate microorganisms and processes which can operate at or above 40°C in order to minimise the need for expensive cooling and sterilising processes. Idris [8] found that several known thermotolerant yeast and fungal strains gave low biomass yields in a screen for SCP production above 40°C. However, strains isolated from two refineries in Scotland gave better results, with one strain of *S. chevalieri* producing a 40% yield of biomass with respect to sugar utilised, composed of 37% protein and 6% RNA.

Recently, the isolation of strains of *K. marxianus* with exceptional fermentative capabilities at temperatures above 40°C was reported [2]. In this study, the SCP potential of these yeasts grown on sugar cane molasses has been assessed.

MATERIALS AND METHODS

Type cultures of *K. marxianus* var. *marxianus*, CBS 712, syn. *K. marxianus* (SRI 1695) and CBS

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397, syn. *K. fragilis* (SRI 1696), were purchased from the Centraalbureau voor Schimmelcultures (CBS), Delft, The Netherlands. All other yeast strains were isolated from Mackay district sugar mill process streams and identified as *K. marxianus* var. *marxianus* (P.J. Anderson, M.Sc. Thesis, James Cook University of North Queensland, 1986).

Sugar cane molasses was collected from Racecourse Co-operative Sugar Mill. The molasses had the following composition: sucrose, 34.90% (w/w); reducing sugars, 10.02% (w/w); dissolved solids, 78.84% (w/w); ash 14.62% (w/w). (Analyses were performed as described in Ref. 4.)

SCP molasses media. Molasses was diluted to give a final concentration of 5% (w/v) sugars. The following nutrients were added (composition expressed as % w/v): 0.5% $(NH_4)_2SO_4$, 0.3% KH_2PO_4, 0.1% yeast extract (Difco). The molasses and nutrient solution were autoclaved separately. The pH after mixing was 5.5. Clarified molasses was prepared by autoclaving 20% (w/v) sugar molasses for 15 min, centrifuging at 16000 × *g* and discarding the centrifuged solids before dilution, autoclaving and addition of nutrients as for unclarified molasses medium.

Syrup YEP agar. Syrup YEP agar had the following composition (expressed as w/v): 0.3% $(NH_4)_2PO_4$, 0.3% KH_2PO_4, 1.0% yeast extract (Difco), 0.5% Bacto peptone (Difco), 1.4% 70 Brix cane syrup, 2.0% agar, distilled H_2O to 1 liter.

HPLC analyses. Glucose, fructose, glycerol and ethanol concentrations were determined by high performance liquid chromatography (HPLC). A Waters (Waters Associates Inc., Melford, U.S.A.) HPLC system was used. A Shodex (Showa Denko K.K., Tokyo, Japan) S-801 IONPAK Carbohydrate column (8 × 500 mm) was used to analyse the culture supernatants. Degassed, 0.2-μm-filtered, glass-distilled water (pH 7.0, 0.005 M NaOH) was used as the eluent for the column at 50°C. All samples were filtered (0.2 μm) before dilution and analysis.

An initial screen selected 14 strains which grew in 1 day at 45°C in test tubes containing 10 ml of 1% (w/v) glucose/0.67% (w/v) yeast nitrogen base (Difco). Cultures were inoculated using a turbidometrically standardised concentration of 2-day-old cells. Strain 974 was shown to produce 6.26% (w/v) ethanol from 15% (w/v) glucose at 45°C in 24 h [2]. Despite its comparatively slow growth at 45°C in this screen, 974 was included because of its importance in ethanol production at high temperatures.

The 15 thermotolerant strains (Table 1) chosen along with the type strains 1695 and 1696 were then screened in 5% sugar molasses broth. A loopful of cells grown for 24 h at 35°C on syrup YEP was used to inoculate starter cultures. Starter cultures (30 ml) consisting of 5% sugar molasses in 125 ml flasks were incubated for 18 h at 140 rpm in a reciprocating shaker water bath at 35°C. A 10 ml aliquot of this starter culture served to inoculate 90 ml of the 5% sugar molasses in 250 ml flasks which were incubated at 120 rpm and 45°C for 24 h. Growth was followed by measuring the turbidity of 1:25 diluted samples in an HACH Ratio Turbidimeter (model 18900). After 24 h incubation, cells were centrifuged at 16000 × *g*, washed twice with distilled water and freeze-dried. The dry weight yield was calculated from the freeze-dried weight, the accuracy of which was checked by vacuum oven drying overnight at 37°C.

Cell samples for amino acid analysis were prepared using 40°C as the final incubation temperature and clarified molasses as the carbon source. Strains were cultured in 5% sugar clarified molasses (250 ml) in 500 ml flasks shaken at 100 rpm and starter cultures (50 ml) were grown up for 18 h at 35°C, 120 rpm in 250 ml flasks. Cells were harvested by centrifuging at 16000 × *g*, washing twice in distilled water and freeze-drying. Amino acid analyses were carried out by Dr. A.C. Kondos (Queensland Agricultural College, Lawes) using a Yanagimoto Amino Acid Analyser Model LC-5 (Yanagimoto Manufacturing Co. Ltd., Japan). Unfortunately, tryptophan could not be determined with the available procedure.

RESULTS

The results in Table 1 represent the screen for protein production on molasses (5% w/v sugars) at

Table 1

45°C single cell protein screen

Results are presented of analyses on cultures grown in N, P and yeast extract-supplemented 5% (w/v) sugar molasses broth, agitated at 120 rpm for 24 h.

Strain	Cell dry.wt.[a] yield ($g \cdot l^{-1}$)	Cell yield, g^{-1} sugars utilised (% w/w)	Growth rate (h^{-1})	Crude protein, N × 6.25 (% w/v)	24 h HPLC analysis[b]	
					ethanol (% w/v)	glycerol (% w/v)
953	4.94	9.88	0.20	49.25	1.72	0.22
955	4.93	9.86	0.23	50.00	1.65	0.20
957	4.73	9.46	0.21	49.44	1.68	0.19
959	4.67	9.34	0.19	48.31	2.18	0.20
775	3.67	7.34	0.22	55.88	1.55	0.19
777	4.52	9.04	0.20	45.44	1.73	0.20
970	4.51	9.02	0.27	50.50	1.60	0.18
971	5.00	10.00	0.24	40.38	1.77	0.20
973	4.85	9.70	0.26	44.88	1.73	0.19
979	4.85	9.70	0.22	45.81	1.99	0.17
779	4.71	9.42	0.19	44.81	1.63	0.18
916	4.82	9.64	0.22	44.56	1.60	0.25
978	5.14	10.28	0.21	43.75	1.50	0.19
952	5.41	10.82	0.23	43.94	1.51	0.16
974	4.40	8.80	0.16	45.01	1.84	0.30
1696	4.19	8.38	0.22	48.81	1.69	0.17
1695	4.34	8.68	0.19	49.94	1.97	0.17

[a] Freeze-dried weight.

[b] Supernatant analysed by HPLC using a Shodex S-801 column. (Sugars were not detected in the 24 h supernatant.)

45°C. The highest dry weight yield was shown by 952. However, 952 had a comparatively low yield of crude protein, 43.94%. All strains completely utilised the carbon source in 24 h. The cell yield (g^{-1} sugars utilised) was low for all strains, and varied from 7.34% (w/w) for 775 to 10.82% (w/w) for 952.

Selected strains were grown in clarified molasses in an attempt to eliminate interference from salts and amino acids in the molasses during the amino acid determination (Table 2). The amino acid compositions of 974 cells grown at 35°C, 40°C and 45°C were compared. There were no great variations in the amino acid profiles at these temperatures. None of the strains produced large proportions of the sulphur-containing amino acids, cystine and methionine. The cell yields of all strains at 45°C and 40°C were low (< 10% (w/w) sugars utilised).

DISCUSSION

At 45°C cell yield from all strains screened for single cell protein production was low (Table 1). Only 4–5.4 $g \cdot l^{-1}$ (dry wt.) biomass was produced from 5% sugar molasses (clarified and unclarified). The concentrations of ethanol produced, 1.50% (w/v) for 978 to 2.18% (w/v) for 959, indicated that growth was largely fermentative under the experimental conditions, with energy derived primarily via production of ethanol and carbon dioxide. In addition, cell maintenance requirements at such high temperatures would be much higher than at lower temperatures and aerobic metabolism would be limited due to a lower concentration of dissolved oxygen at the higher temperatures.

The data did not indicate a high cell yield/sugars utilised ratio in any of the strains examined. The

Table 2

Amino acid composition and growth characteristics of selected strains

Amino acid composition is expressed as a percentage of the freeze-dried cell weight. Yeasts were cultured in 5% (w/v) sugar clarified molasses supplemented with N, P, and 0.1% yeast extract, agitated at 100 rpm (reciprocal) at 40°C except where indicated for 974 in parentheses.

Amino acids (% w/w dry wt. yield)	955	953	952	1695	1696	971	974 (35°C)	974	974 (45°C)
Aspartic acid	5.59	5.24	4.48	5.56	5.50	5.10	5.34	5.56	5.02
Threonine	2.72	2.46	2.23	2.65	2.54	2.59	2.44	2.40	2.46
Serine	2.87	2.67	2.44	2.79	2.66	2.56	2.36	2.40	2.57
Glutamic acid	6.70	7.21	5.80	6.77	6.46	6.24	6.81	6.50	4.90
Proline	1.94	1.98	1.67	1.76	1.85	1.79	2.17	1.81	2.09
Glycine	2.32	2.24	2.12	2.33	2.22	2.32	1.98	2.05	1.99
Alanine	4.49	4.30	3.20	4.59	4.39	3.60	3.52	4.13	4.42
Cystine	0.27	0.23	0.23	0.28	0.45	0.23	0.23	0.23	0.21
Valine	2.39	2.41	2.36	2.53	2.52	2.46	2.37	2.18	2.55
Methionine	0.62	0.54	0.60	0.60	0.70	0.59	0.60	0.47	0.60
Isoleucine	2.06	1.97	1.89	2.17	2.12	2.10	1.93	1.92	1.93
Leucine	3.49	3.04	2.90	3.36	3.48	3.32	3.12	3.12	3.04
Tyrosine	1.60	1.36	1.43	1.79	1.44	1.70	1.36	1.37	1.64
Phenylalanine	2.09	1.84	1.67	1.84	1.93	2.00	1.75	2.07	1.95
Lysine	3.67	3.28	4.01	3.36	3.63	3.85	3.53	3.48	3.61
Histidine	0.91	0.83	0.91	0.85	0.82	0.97	0.87	0.91	0.89
Arginine	2.05	2.02	2.01	2.06	2.14	2.24	2.17	2.05	1.98
Total amino acids	45.78	43.62	39.95	45.29	44.85	43.65	42.55	42.67	41.85
Dry wt.[a] yield ($g \cdot l^{-1}$)	4.94	4.83	4.44	4.89	4.95	4.75	4.95	5.15	4.52
Cell yield, g^{-1} sugars (% w/w)	9.88	9.66	8.88	9.78	9.90	9.50	9.90	10.30	9.04
Growth rate (μh^{-1})	0.30	0.44	0.35	0.35	0.33	0.40	0.29	0.20	0.20
Crude protein, N × 6.25 (% w/w)	49.84	46.83	45.85	49.46	50.54	49.49	45.43	47.74	44.87
Ethanol[b] (% w/v)	1.81	1.91	1.23	2.16	2.23	1.88	1.88	2.16	2.17
Glycerol[b] (% w/v)	0.31	0.39	0.41	0.26	0.33	0.36	0.36	0.35	0.30

[a] Freeze-dried weight.

[b] HPLC analysis of 24 h culture supernatant. Sugars were not detected.

highest ratio was that for strain 952, 10.82% g dry wt. $\cdot g^{-1}$ total sugars. This was low in comparison with values reported for *K. marxianus* strains in the literature; 36–40% by Apaire et al. [3] for 0.5% inulin, 24–53% by Gomez et al. [6] for 2% (w/v) sugar molasses, and high in comparison with two strains of *S. lactis* (syn. *K. marxianus* var. *lactis*), 4.5 and 6.0% by Mahmoud et al. [11]. Idris and Berry [9] isolated thermotolerant yeasts for the production of biomass on molasses (1% w/v) medium. Several strains which gave biomass yields of 30–40% (biomass dry wt./sugar dry wt.) at 40°C were isolated and identified. One strain of *K. fragilis* gave a 33% yield at 40°C.

Forage and Righelato [5] listed *C. utilis*, the most widely accepted yeast strain for SCP production, as producing a cell yield of 50% (biomass/carbohydrate). It appears that the *K. marxianus* var. *marxianus* strains examined in this work have exhibited anaerobic dissimilation of available sugars with ethanol and carbon dioxide as major products and a consequent low biomass yield, despite vigorous agitation of the growth medium.

The Crabtree effect results in fermentative

growth of some yeasts (e.g., *S. cerevisiae*) even with vigorous oxygenation when the glucose concentration and/or the growth rate exceed critical values. For example, fermentative growth of *S. cerevisiae* was claimed to commence when the glucose concentration in the growth medium exceeded 70 mg $\cdot l^{-1}$ [14].

Yeast protein has been shown to contain all of the amino acids essential in human nutrition but is characteristically low in the sulphur-containing amino acids, cystine and methionine. The yeasts listed in Table 2 had concentrations of amino acids comparable to those previously reported for strains of *Saccharomyces, Kluyveromyces* and *Candida* [3,12]. The most promising strains (*S. cerevisiae* and *C. utilis*) found by Abdel-Hafez et al. [1] showed similar concentrations of lysine but higher levels of cystine and methionine than the SRI strains. The levels of lysine, cystine and methionine obtained by Gomez et al. [6] were comparable to those observed in the strains examined in this report. On the basis of yields and toxicological aspects, Gomez et al. [6] recommended microbial biomass grown from strains of *K. fragilis, C. pseudotropicalis* and *S. cerevisiae* as being useful in supplementing human diets.

The future of microbial biomass as a major or sole protein source for man is doubtful, although microbial biomass may be useful in supplementing human and animal food low in essential amino acids. Despite such claims, it is difficult to foresee large-scale production of microbial protein unless there are massive improvements in technology. The applicability of SCP to Third World food problems is greatly limited by the high costs and energy requirements [10] which Forage and Righelato [5] have suggested to be primarily the costs of the carbon substrate and oxygenation.

Although a potential for thermotolerant strains of *K. marxianus* from sugar mills to be used in ethanol production has been established [2], further experimentation to optimise substrate and nutrient concentrations and oxygenation rates will be necessary before the value for SCP production can be gauged.

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