Isolation and identification of yeast strains capable of producing single cell protein from whey in co-cultures with *Saccharomyces cerevisiae*

Hassan Moeini[1,2], Sadeq Vallian[1] and Iraj Nahvi[1]

[1]Department of Biology, Faculty of Science, Isfahan University, Isfahan, I.R. Iran, [2]Department of Biology, Faculty of Science, Shahid Chamran University, Ahwaz, I.R. Iran.

Abstract
In this study, twenty-five whey samples collected from dairy industries in the city of Isfahan. The samples were cultured on malt extract broth (MEB) and yeast extract glucose chloramphenicol agar (YGCA) media. Eleven yeast strains (designated M1 to M11) were isolated from the culture. The strains were identified by their morphological and physiological properties. Beta-galactosidase activity in the yeast strains showed that a strain of K. lactis designated as M2 had highest enzyme activity (up to 8103 EU/ml). The isolated yeast strains were examined for their ability in reduction of the biological oxygen demand (BOD). The results demonstrated a high level of reduction in the M2 strain. This strain was also found to have highest level of single cell protein (SCP), production (up to 11.79 g/l dry mass cell). The co-culture of the isolated yeast strains with Saccharomyces cerevisiae resulted in the highest biomass yield up to 22.38 g/l dry mass cell and significant reduction in initial BOD. Together, the data showed that the isolated yeast strain could be of valuable application in bioconversion of whey.
Keywords*: Candida versatilis, Beta-galactosidase, Kluyveromyces lactis, Kluyveromyces marxianus, SCP, Whey.*

INTRODUCTION

Whey is the aqueous fraction of milk generated as a by-product of cheese manufacturing which is produced in large amounts. The main part of cheese whey is lactose, present at a concentration of about 4.5-5% (Rohm *et al.*, 1992; Roostita and Fleet, 1996). Other components are protein, salts and vitamins that are present in minor amounts. The low concentration of these components makes their recovery uneconomical. Because of its high organic content with high biological oxygen demands (BOD), 40000-60000 ppm, dumping whey directly to the environment causes serious contamination problems (Cristiani-Urbina *et al.*, 2000; Roostita and Fleet, 1996). To overcome this problem, bioconversion of whey into single cell protein, (SCP), or ethanol has been performed in several countries (Gonzales, 1996; Irvin and Hill, 1985; Marshall, 1987 and Mawson, 1994). SCP could be produced from whey, with employing of yeasts from different species including *Kluyveromyces*, *Candida*, and *Trichosporon*, which are normally capable of metabolizing lactose (Castillo, 1990; Fleet, 1990; Fleet and Main, 1987; Galvez *et al.*, 1990; Seiler and Busse, 1990). However, it has been observed that in the air limited cultures of *Kluyveromyces fragilis*, and *K. lactis* a change in the cellular metabolism from oxidative to a mixed oxidative-fermentative state can be occurred. These changes could result in production of by-metabolic products such as alcohol, aldehydes, esters, etc., which reduce the yield of biomass on whey (Beausejour *et al.*, 1981; Moresi *et al.*, 1989, 1990 and Pigache *et al.*, 1992). It has been shown that a co-culture of *Kluyveromyces* strains and *S. cerevisiae* could overcome these undesired effects (Pigache *et al.*, 1992).

In this study, a number of yeast strains with lactose fermentation ability were isolated and identified. The ability of the strains for consumption of lactose, SCP production and BOD removal was evaluated. Furthermore the isolates were co-cultured in order to increase the yield of SCP.

Correspondence to: **Sadeq Vallian, Ph.D.**
Tel/Fax: +98 311 7932456
E. mail: svallian@sci.ui.ac.ir

MATERIALS AND METHODS

Sampling and isolation of yeast strains

Twenty-five samples including whey, yoghurt and cheese were collected from dairy producing factories in Isfahan. The samples collected in sterile 200 ml bottles and transferred to laboratory in a cooler box. The yeasts were enriched by inoculation of 5 ml (or 5 gr) of the sample in 50 ml of Malt Extract Broth (MEB), containing 0.1 g/l chloramphenicol. The incubation was performed at 25°C for 24 h with constant shaking of 180 rpm. The yeast cells in media was examined using light microscopy. The yeast strains were isolated on spread plates of yeast extract glucose chloramphenicol agar (YGCA) after making serial dilutions. The plates were incubated at 25°C for 72 h. Colonies with distinct morphological differences were selected and purified by streaking on potato-dextrose agar (PDA) (Bainotti *et al.*, 1987).

Identification of yeast strains

To identify the yeast strains capable of lactose fermentation, the isolated yeasts were cultured in Durham tubes containing 2% (w/v) lactose (Fleet and Main, 1987). Then, the positive yeast strains for lactose fermentation were identified using the standard taxonomic key outlined as described (Kutzman and Fell, 1998).

Liquid assimilation of carbon compounds and nitrogen compounds were performed as described by Fleet and Main (1987). The carbon compounds tested were galactose, sucrose, maltose, cellulose, trehalose, melibiose, raffinose, inuline, D-xylose, L-arabinose, D-ribose, L-rhamnose, glycerol, D-mannitol, citrate and inositol. The procedure for liquid assimilation was similar to the assimilation of carbon compounds test except that the yeast nitrogen base was replaced by yeast carbon base (Difco, Germany). The nitrogen compounds tested were nitrate and L-lysine (Fleet and Main, 1987).

For determining the ability of yeast growth at 37°C and 40°C, they were cultured in a medium containing 2% (w/v) glucose-peptone-yeast extract broth (Cristiani-Urbina *et al.*, 2000; Fleet and Main, 1987). The samples were inoculated with actively growing yeast culture and incubated at 37°C and 40°C for 1-2 h at constant shaking of 180 rpm. A positive reaction was detected by observation of turbidity in the solution (Cristiani-Urbina *et al.*, 2000).

The ability of the isolated yeast strains at high concentration of sugar was tested asdescribed by Fleet and Main (1987). Hydrolysis of urea was examined by using commercially available Christensen's urea agar base (Merck, Germany). The slant was inoculated from an actively growing yeast culture and incubated at 25°C for 4 days. The ability of hydrolysis of urea was detected by development of a pink color in the slant medium (Deak and Beuchat, 1996).

Measurement of beta-galactosidase activity

O-Nitrophenol β-D-Galactopyranoseide (ONPG) assay was applied for calculation of beta-galactosidase enzyme activity in the yeast strains as previously reported (Gunther and Burger, 1982). Briefly; the isolated strains were inoculated in YEPD medium and cheese whey, and incubated at 25°C for 12-18 h. One ml of yeast cultures (approximately 10^7 cells) at OD_{600} was span down. Then, the cells were washed in 1ml cold Z buffer (0.06 M Na_2HPO_4, 0.04M NaH_2PO_4, 0.01M KCl and 0.001M $MgSO_4$). The activity of the beta- galactosidase enzyme was measured in the presence of ONPG at OD_{420} nm and the Miller units was calculated as described.

SCP production and BOD removal from whey

Production of SCP was measured on the bases of weight of dry biomass (g/l) in triplicates. Fresh whey collected in sterile bottles and boiled at 100°C for 15 min. Whey was cooled and sedimented proteins were collected by filtration. One liter of the limpid liquid obtained (greenish yellow) was sterilized at 115°C for 10 min and then inoculated by an actively growing culture. The medium was incubated at 25°C for 48 h with constant shaking at 180 rpm. After incubation, the biomass of the yeast cells was prepared by centrifugation at 4000 ×g. The biomass was dried and then weighed (Jakobsen and Narvhus, 1996 and Litchfield, 1983). The effect of nitrogen supplementation on the yield of SCP was studied by using ammonium sulfate as the nitrogen source. After sterilization, the medium was inoculated with the yeast strains and aired as mentioned above (Galvez *et al.*, 1990 and Litchfield, 1983).

Selected isolates co-cultured with *S. cerevisiae* (Isfahan University, Department of Microbiology, Yeast strains collection) for treatment of cheese whey and SCP production. The selected strains together with *S. cerevisiae* were inoculated into YEPD medium and incubated at 25°C for 18-24 h with shaking at 200 rpm. After preparation of whey, 4 ml of yeast medium was added to the whey medium and incubated (as described) (Cristiani-Urbina *et al.*, 2000 and Carlotti *et al.*, 1991).

Table 1: Identification of yeasts isolated from dairy producer centers in the city of Isfahan.

Conventional name of Yeast	Fermentation						Assimilation																Growth in 50% Glucose	Growth in 37°C	Growth in 40°C	Urase Activity	Yeast Species
	Glucose	Galactose	Sucrose	Maltose	Raffinose	Trehalose	Galactose	Sucrose	Maltose	Cellobiose	Trehalose	Melibiose	Raffinose	Inuline	D-Xylose	L-Arabinose	D-Ribose	L-Rhamnose	Glycerol	D-Mannitol	Citrate	Inositol	Nitrate	L-Lysine			
M1	+	+	+	-	+	-	+	+	-	+	+	-	+	+	+	-	-	-	+	+	-	-	-	-	+	+	-	*K.Lactis*
M2	+	+	+	-	+	-	+	+	+	+	+	-	+	-	+	-	-	-	+	+	-	-	-	-	+	-	-	*K.Lactis*
M3	+	+	+	-	+	-	+	+	-	+	+	-	+	+	+	+	-	-	+	-	-	-	-	+	ND	+	-	*K.Marxianus*
M4	+	+	+	-	+	-	+	+	-	+	-	-	+	+	+	+	-	-	+	+	-	-	-	+	ND	+	-	*K.Marxianus*
M5	+	+	+	+	+	-	+	+	+	+	+	-	+	-	-	-	-	-	+	+	-	-	-	-	+	-	-	*K.Lactis*
M6	+	+	+	-	+	-	+	+	+	+	+	-	+	-	-	-	-	-	+	+	-	-	-	-	+	-	-	*K.Lactis*
M7	+	+	+	-	+	-	+	+	-	+	+	-	+	-	+	-	-	-	+	+	-	-	-	-	+	-	-	*K.Lactis*
M8	+	+	+	-	+	-	+	+	-	-	-	-	+	+	+	+	-	-	+	+	-	-	-	+	ND	+	+	*K.Marxianus*
M9	+	+	+	-	+	-	+	+	+	+	+	-	+	-	-	-	+	-	+	+	-	-	+	+	+	-	-	*C.Versatilis*
M10	+	+	+	-	+	-	+	+	-	+	+	-	+	-	-	-	-	-	+	+	-	-	-	+	-	-	-	*K.Lactis*
M11	+	+	+	-	+	-	+	+	-	-	-	-	+	+	+	-	+	-	+	-	-	-	-	+	ND	-	-	*K.Marxianus*

ND= Not determined

(-) Sign denoted observe and (+) shows presence

Biological oxygen demand (BOD) removal in pure and mixed yeast culture was assayed as follows: The pure cultures of the M2 (*K. lactis*) and M11 (*K. marxianus*) were used for BOD removal in whey. In each assay, 200 ml of fresh whey was boiled at 100°C for 15 min and the denatured proteins were removed and then inoculated with strains and incubated at 25°C for 48 h with shaking at 200 rpm. After incubation, the supernatant was used for BOD assay. The co-culture of these strain and *S. cerevisiae* was also used to enhanced BOD removal (Carlotti *et al.*, 1991).

RESULTS

In this study, 30 different yeast strains were isolated by using malt extract broth (MEB) containing 0.1 gl^{-1} chloramphenicol and yeast extract glucose chloramphenicol agar (YGCA). The isolates were examined for lactose fermentation ability. Among them, 11 strains (M1-M11) were found capable of lactose fermentation (Table 1). These strains were identified by morphological and physiological properties using the standard taxonomic key as outlined (Kutzman and Fell, 1998; Gadaga *et al.*, 2000). These strains were further identified using several chemical tests including fermentation of different sugars, liquid assimilation of carbon and nitrogen compounds, growth at 37 and 40°C, growth in 50% glucose, and their urase activity. As shown in Table 1, six isolates were identified as *Klyveromyces lactis.* Four strains were identified as *Klyveromyces marxianus.* Among the strains identified one classified as *Candida versatilis*.

Enzyme activity of beta-galactosidase in yeast strains was measured. Among 11 yeast strains, the M2 strain (*K. lactis*) was found to have the highest enzyme activity, 8183 unit/ml. Also the M5 strain (*K.lactis*) and M11 strain (*K. marxianus*) were showed high enzyme activity, 5487 and 5357 unit/ml, respectively. In cheese whey, as culture medium, enzyme units of the M2, M5 and M11 strains were 5266, 5020 and 4642 unit/ml, respectively (Table 2).

Table 2: Beta-galactosidase activity in the isolated yeast strains.

Isolate	Species	Beta-galactosidase activity (EU/ml)*	
		YEPD medium	Using whey medium
M2	*K. Lactis*	8103	5266
M5	*K. Lactis*	5487	5020
M11	*K. Marxianus*	5357	4642

*The beta-galactosidase activity was measured using ONPG and calculated as follows: Units=1000 $\times OD_{420}$ / volume (1ml) $\times$ time (min) $\times$ OD_{600} (Gunther and Burger, 1982).

Effects of nitrogen supplementation on the biomass yield

The single cell protein (SCP), production by the isolated yeast strains was studied (see above). After preparation of cheese whey, inoculation of yeast strains under appropriate temperature, the biomass yield of the pure culture of the isolated yeast strains was measured (Table 3). In whey medium without any supplementation, the M2 (*K. lactis*), M11 (*K. marxianus*) and M5 (*K. lactis*) strains with 11.79, 11.54 and 11.09 g/l dry biomass yield had, respectively, the most SCP production (Table 3). Supplementation of the media with 0.8 g/l of ammonium sulfate resulted in a significant increase in biomass yield (Table 3). Under such conditions, the M11 (*K. marxianus*) and M2 (*K. lactis*) strains were found to have the highest biomass yield, up to 15.75 and 15.35 g/l, respectively.

Improvement of biomass yield and BOD removal using co-culture

Co-cultures of isolated yeast strains (M2, M5, M6 and M11) with *S. cerevisiae* were used (Table 3). The biomass yield of *S.cerevisiae* and the M2 strain (*K. lactis*) co-culture showed a significant increase from 11.79 to

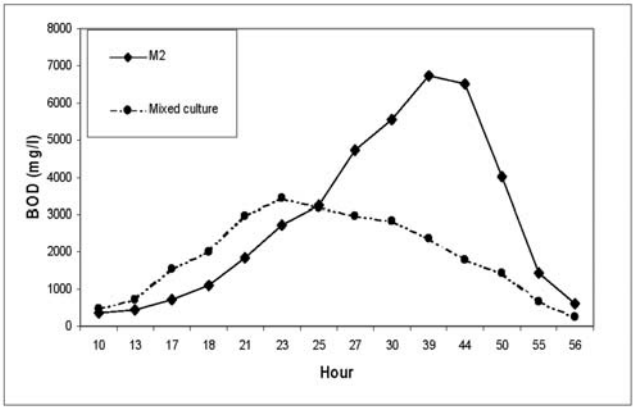
Table 3: Analysis of the amount of SCP production in isolated yeast strains.

Isolate	Species	Biomass yield (g/l) in whey as culture medium*		
		No supplementation	With ammonium sulfate (0.8 g/l)	Co-culture of yeast strains & *S. cerevisiae* + Ammonium Sulfate
M2	*K. lactis*	11.79 ± 0.12	15.35 ± 0.25	22.38 ± 0.30
M5	*K. lactis*	11.00 ± 0.17	15.00 ± 0.34	17.11 ± 0.32
M11	*K. marxianus*	11.54 ± 0.10	15.75 ± 0.14	19.58 ± 0.20

*The numbers indicate the average of three repeats.

15.35 g/l. Supplementation of the cultures of the isolated yeasts with $(NH_4)_2SO_4$ as nitrogen source resulted in a further increase in the biomass production to 22.38 g/l (Table 3). In this study the BOD removal was also examined using pure cultures of M2 (*K. lactise*) and M11 (*K. marxianus*) as well as mixed cultures of each yeast strain with *S. cerevisiae*. A greater BOD removal efficiency (88.5%) was obtained using co-culture of M2 strain (*K. lactis*) and *S. cerevisiae* (Fig. 1).

Figure 1: BOD removal using pure culture of M2 strain (*K. lactis*) and co- culture of M2 strain and *S. cerevisiae.* The selected strains and *S. cerevisiae* were inoculated into YEPD medium and incubated at 25°C for 18-24 h with shaking at 200 rpm.



DISCUSSION

In this study 30 different yeast strains were isolated among which 11 strains were capable of lactose fermentation (M1-M11) (Table 1). These strains were identified using several chemical tests. Of these isolates, six were identified as *Klyveromyces lactis* and four strains as *Klyveromyces marxianus.* Among the strains identified, one classified as *Candida versatilis*. In comparison to other studies, it seems that *K. lactis, K. marxianus* and its anamorph, *C. kefyer,* are the most predominant and important yeast species in milk (Fleet, 1990; Gadaga *et al.*, 2000; Seiler and Busse, 1990). As for the other yeast strains isolated and identified in this study, *Candida versatilis* has been reported in yogurt, cheese and other dairy products (Gadaga *et al.*, 2000; Suriyarachchi and Fleet, 1981). *K. marxianus and K. Lactis* were used in several biotechnology applications (Klaus, 1996). The thermotolerance of *K. marxianus* which was also identified among the yeasts isolated in the present study, could be used in very rapid processes of ethanol production, which might compensate for its lower tolerance to ethanol as compared to *S. cerevisiae*. *Kluyveromyces fragilis* (*K. marxianus)* strains are the yeasts that have been most widely studied for the production of yeast biomass from whey (Carlotti *et al.*, 1991 and Castillo, 1990) and large-scale processes for *Kluyveromyces* biomass production have been in operation for several years (Carlotti *et al.*, 1991). *K. Lactis* has been used for its industrial potential in the production of beta-galactosidase enzyme which could be used to reduce lactose content in the milk (Suarez *et al.*, 1995).

As shown in Table 2, among 11 yeast strains, the M2 strain (*K. lactis*) was found to have the highest enzyme activity, up to 8183 unit/ml, which is relatively rare among yeast strains isolated (Gunther and Burger, 1982). Also the M5 strain (*K. lactis*) and M11 strain (*K. marxianus*) showed a relatively high level enzyme activity, 5487 and 5357 unit/ml, respectively. In cheese whey, as culture medium, the enzyme activity in M2, M5 and M11 strains was 5266, 5020 and 4642 unit/ml, respectively.

As illustrated in Table 3, in whey medium without any supplementation, the M2 (*K. lactis*), M11 (*K. marxianus*) and M5 (*K. lactis*) strains had the most SCP production capacity with 11.79, 11.54 and 11.09 g/l dry biomass yield, respectively. Amount of SCP production can be improved by adding of ammonium sulfate as nitrogen supplementation (Cristiani-Urbina *et al.*, 2000). Ammonium sulfate was found to significantly affect biomass yield, as a result the produced biomass of M11 strain increased from 11.54 g/l, in whey without supplementation, to 15.75 g/l, in presence of nitrogen supplementation.

A number of studies showed that when *K. lactis* and *K. marxianus* (*K. fragilis*) were grown in whey, could form extracelluar metabolites such as ethanol, esters, aldehydes etc. (Beausejour *et al.*, 1981 and Moresi *et al.*, 1989). These intermediate compounds can be metabolized by some other yeast strains. In this way, to increase the biomass yields, the mixed culture of yeast strains has been studied (Carlotti *et al*., 1991 and Cristiani-Urbina *et al*., 2000). Similarly, we used mixed culture of isolated yeast strains (M2, M5, M6 and M11) with *Saccharomyces cerevisiae.* Table 3 shows that the biomass yields of the co-cultures of the selected strains and *S. cerevisiae* were significantly greater as compared to that measured in single-strain cultures. The biomass yield obtained under this condition was greater than those reported for *K. marxianus* (Bainotti *et al.*, 1987), *T. cremoris* (Litchfield, 1983), *K. fragilis* (Beausejour *et al.*, 1981), for the co-cultures of *K. fragilis, K. lactis, Torulopsis* (Pigache *et al.*,

1992) and *C. kefyr* and *C. valida* (Carlotti *et al.*, 1991). The biomass of *Kluyveromyces* species can be used as dietary supplement in feeding domestic animals (Irvine and Hill, 1985).

Study of the BOD removal using pure cultures of M2 (*K. lactise*) and M11 (*K. marxianus*) strains and mixed cultures of these yeast strains with *S. cerevisiae* showed a significant increase in BOD removal by mixed culture of M2 and M11 strains with *S. cerevisiae*, A greater BOD removal efficiency (up to 88.5%) was obtained using co-culture of M2 strain (*K. lactis*) and *S. cerevisiae* (Fig. 1). The BOD removal achieved for the mixed culture was greater than those reported for *K. marxianus* (Pigache *et al.*, 1992).

Since the *S. cerevisiae* could not grow in lactose medium, it might have consumed some of the extracellular metabolites produced during the growth of *Kluyveromyces* species. In order to confirm this ability, the M2 and M11 strains were cultured on whey. The data showed that isolated *Kluyveromyces* species could be valuable strains for removal of whey pollutants, treatment of lactose intolerance by reducing the lactose content of milk, SCP and beta-galactosidase production from whey.

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