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Ethanol from lactose in salted cheese whey by recombinant *Saccharomyces cerevisiae*

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Abstract Seven yeast recombinants were selected from 57 fusant colonies on the basis of higher DNA content, nuclear diameter and ethanol yield compared to parental *Saccharomyces cerevisiae* ATCC 4126 and *Kluyveromyces lactis* CBS 683. Six recombinants out of the above colonies revealed growth on lactose and sucrose, indicating that they are *S. cerevisiae* with transformed β-galactosidase systems. The fusant colonies were investigated with respect to their capacity to convert lactose in salted cheese whey into ethanol. Among these recombinants that showed high tolerance towards sodium chloride and higher ethanol yield than lactose fermenting parental *K. lactis* CBS 683, SK-1 exhibited high tolerance up to 4 g dl^{-1} sodium chlonride with an ethanol yield of 4.66 ml dl^{-1} (v/v) , SK-23 tolerated 6 g dl^{-1} sodium chloride with an ethanol yield of 4.14 ml dl^{-1} (v/v) and SK-26 showed resistance towards 8 g dl^{-1} sodium chloride and give an ethanol yield of 3 ml dl^{-1} (v/v).

Key words *Saccharomyces cerevisiae* · Lactose · Ethanol production · Cheese whey · Recombinant yeast strains

Introduction

Cheese whey is the major by-product of the dairy industry. In 1974, 32.5 billion pounds of whey were produced, over half of which was disposed of as waste [1]. This represents a pool of 1.6 million pounds of lactose, which if converted into usable food products could be of sizeable monetary value to the dairy industry. However, disposal of whey without expensive sewage treatment can represent a source of water pollution. Industrial processes for ethanol, biomass, organic acid and amino acid production from whey have been developed [2].

Lactose is one of the several least expensive carbon sources for ethanol production. Although earlier attempts were made to use lactose for alcohol production [3], the major problem was the inability of *Saccharomyces cerevisiae* to ferment lactose. The relatively small number of other microorganisms which are capable of fermenting lactose usually give a low yield of ethanol. *Kluyveromyces fragilis* is known to ferment lactose [4]. However, it has been shown that only a fraction of the available lactose is converted to alcohol, possibly due to alcohol inhibition [5]. *S. cerevisiae* cells can grow on lactose after a preliminary enzymatic or chemical hydrolysis. However, the presence of both glucose and galactose at fairly high concentrations can result in diauxic growth; first there is a fermentative growth on glucose and then a slower growth on galactose [6]. Thus, both the overall yield and the production of ethanol are low. To overcome the above-mentioned problems, a genetically engineered strain of *S. cerevisiae* that expresses β-galactosidase activity was reconstructed for the bioconversion of lactose in whey into chemicals.

Successful attempts in that respect yielded the recombinant yeast strains that were prepared by intergeneric protoplast fusion of *S. cerevisiae* and *K. fragilis* [7–9]. The obtained recombinants revealed not only prototrophic characteristics on minimal media but were also capable of producing high yields of ethanol from lactose in lactose-dependent media. However, it was reported that on using the recombinant hybrids for whey conversion into ethanol, the maximum yield did not exceed more than 3.4 ml dl^{-1} (w/v) after 48 h.

Utilization of unsalted whey as a fermentation substrate to produce biomass and organic acids has been described [3]. In Egypt, most produced milk is converted to cheese after being salted with sodium chloride up to 6 g dl^{-1} (w/v). Thus, the aim of the present work is to find an appropriate yeast strain that can convert lactose in salted cheese whey into ethanol.

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Materials and methods

Microorganisms

Yeast strains used in the present investigation were recombinant hybrids obtained from intergeneric protoplast fusion of industrial strains *S. cerevisiae* ATCC 4126 [cycloheximide$^-$, imazilil$^+$], fermenting sucrose, glucose and fructose, and *K. fragilis* CBS 683 [imazilil$^-$, cycloheximide$^+$], fermenting lactose, glucose and galactose [10]. The parental and recombinant strains were maintained on YEPD medium [11] and stored at 4 °C.

Media

Botham and Ratledge medium. The medium was prepared according to Botham and Ratledge [12], while glucose was sterilized by autoclaving at 121 °C for 30 min and added to the medium to give a final concentration of 50 g dl^{-1} (w/v).

Yeast extract peptone dextrose (YEPD). The medium was prepared as previously described [11], where slants from it were sterilized at 110 °C for 30 min.

Cheese whey medium (CWM). Cow's skimmed milk was obtained by separation of fresh cow's milk (faculty herd), while fresh whey was prepared from skimmed milk by renneting at 37 °C. A whey-containing medium was prepared [13] by adjusting the whey to pH 4.6 with concentrated HCl, heated at 90 °C for 15 min, cooled and centrifuged at 800 *g* for 10 min to remove coagulated proteins. Whey medium containing 4–6 g dl^{-1} (w/v) lactose was supplemented with 0.3 g K_2HPO_4, 0.5 g yeast extract and 0.3 g dl^{-1} (w/v) of 5 mol l^{-1} NH_4OH. The medium was adjusted to pH 4.5 with H_2SO_4, autoclaved for 15 min at 121 °C, cooled to 30 °C and inoculated.

Spore formation and isolation

To obtain haploids (*n*) from (2*n*) diploid parental *K. fragilis* CBS 683 or *S. cerevisiae* ATCC 4126, the yeasts were sporulated after 5 days of incubation at 30 °C on sporulation medium containing 1 g potassium acetate, 0.5 g glucose, 0.1 g yeast extract and 2 g dl^{-1} (w/v) agar. The isolation of spores was carried out by the method of Farahnak et al. [7].

Systemic fungicides mutant selection

Cycloheximide [3-(2,3,5-trimethyl-2-oxocyclohexyl)-2-hydroxyethyl glutarimide] and imazilil [1-β-(alkyloxy)-2,4-chlorophenetylimidazole phosphate] were used for labelling parental yeast strains. The inhibitors were dissolved in methanol to give a final concentration of 1–100 μg ml^{-1} of YEPD medium in order to determine the minimum inhibitory concentration (MIC) of each inhibitor. The mutants of the investigated yeast strains which were resistant to fungicides were isolated by subculturing the yeast at 2–5 times the concentration of the MIC of each systemic fungicide.

Protoplast isolation and fusion

Protoplast isolation was achieved first by growing the labelled resistant mutant spores of each strain until early log phase. A 10^6 cell mass of each spore was inculated in YEPD medium separately at 28 °C for 20 h with shaking. The germinated spores were collected by centrifugation for 10 min. The cells were suspended in the protoplasting solution buffer containing 0.6 M KCl, 10 mmol 2-mercaptoethanol, 50 mmol phosphate buffer (pH 7.5) and 5 mg zymolase (60000) ml^{-1}. The suspension was incubated at 30 °C for 1 h with occasional shaking, and it was checked periodically under the microscope for the formation of protoplasts. Protoplasts were collected and washed at least three times with phosphate buffer containing 0.8 mol sorbitol (pH 7.5). Equal numbers of protoplasts of each strain were mixed and suspended in PEG 6000 (35 g dl^{-1}, w/v) containing 10 mmol $CaCl_2$ and 0.8 mol sorbitol. The suspension was incubated for 20 min at room temperature. The fused cells were washed again with protoplasting buffer, followed by centrifugation. The pellet was diluted with protoplasting buffer. Aliquots of the fusion product were subcultured, embedded on a regeneration osmotically minimal medium (OMM) supplemented with vitamins and 0.6 mol KCl, followed by another layer of OMM containing the two systemic fungicides. The plates were incubated at 28–30 °C for 14–20 days.

Measurement of DNA

Nucleic acid extraction was achieved as reported earlier [14], while the DNA content was determined using the diphenylamine reagent method according to Burton [15].

Nuclear staining and measurement of nuclear diameter

Parental strains and diploids were fixed and stained according to Robinow [16]. The nuclear diameter was measured according to the method of Clutterbuck Roper [17].

Alcohol fermentation

A volume of 50 ml of Botham and Ratledge medium used for ethanol production was transferred into 100 ml fermentation bottles with rubber stoppers equipped with a hypodermic syringe. The bottles were inoculated with a cell suspension of 10^6 cells ml^{-1} and incubated with mild shaking at 30 °C for 96 h.

Ethanol measurement

Ethanol concentration in the fermentation medium filtrates was measured by gas chromatography using a Shimadzu GC-8A (Shimadzu, Japan) equipped with a flame ionization detector. A column 2 m × 2 mm, packed with chromosorb W/AW (60–80 mesh) impregnated with 15 g kg^{-1} carbowax 20 M was used. The temperatures of the injector and detector were maintained at 220 °C and the column oven was operated of isothermally at 130 °C. A sensitivity of 10^2 and a range of 32 were used during the analysis.

Measurement of β-galactosidase activity

A 10^6 cell mass of yeast cells propagated first in Botham and Ratledge medium by incubation at 37 °C for 48 h was harvested by centrifugation at 3000 *g* for 10–15 min. The cell mass was suspended in phosphate buffer (pH 7) and 2 g dl^{-1} (v/v) toluene was added for cell autolysis [13]. Measurement of β-galactosidase activity was carried out according to Mahoney and Adamchuk [18] using *O*-nitrophenyl-β-D-galactopyranoside (ONPG) reagent. One ONPG unit of the enzyme activity was defined as the amount of enzyme which liberates 1 μmol *O*-nitrophenol min^{-1}.

Sugar measurement

The sugar content was determined using the phenol/sulfuric acid reagent method [19].

Results and discussion

The incidence of intergeneric protoplast fusion between *S. cerevisiae* ATCC 4126 and lactose fermenting *K. lactis* CBS 683 was initially confirmed by selection of colonies that showed vigorous growth on stabilized minimal medium containing the MIC of imazilil (7 μg ml^{-1}) for *K. lactis* and cycloheximide (10 μg ml^{-1}) for *S. cerevisiae* as markers for parental strains. Out of 1000 colonies, 57 were chosen and subjected to different

Table 1 Some properties of selected recombinants with elevated ethanol yields compared to parental *Saccharomyces cerevisiae* ATCC 4126 and *Kluyveromyces lactis* CBS 683 grown on 10 g dl^{-1} (w/v) lactose at 30 °C for 96 h (average of three replicates)

Nuclei	DNA content (μg per 10^6 protoplasts of recombinant per parental strains)		Nuclear diameter	Ethanol (ml dl^{-1})	Increase of ethanol over both of the two parental strains
	CBS 683	ATCC 4126	(mm)	(v/v)	(%)
CBS 683			0.00068	4.56	
ATCC 4126			0.00079	0.23	
SK-1	1.65	1.75	0.00140	6.17	28.81
SK-23	1.63	1.53	0.00120	5.97	24.64
SK-26	1.47	1.66	0.00130	7.42	54.91
SK-35	1.87	1.81	0.00130	8.06	68.27
SK-48	0.85	1.29	0.00120	6.97	45.50
SK-53	1.52	2.23	0.00120	6.28	31.11

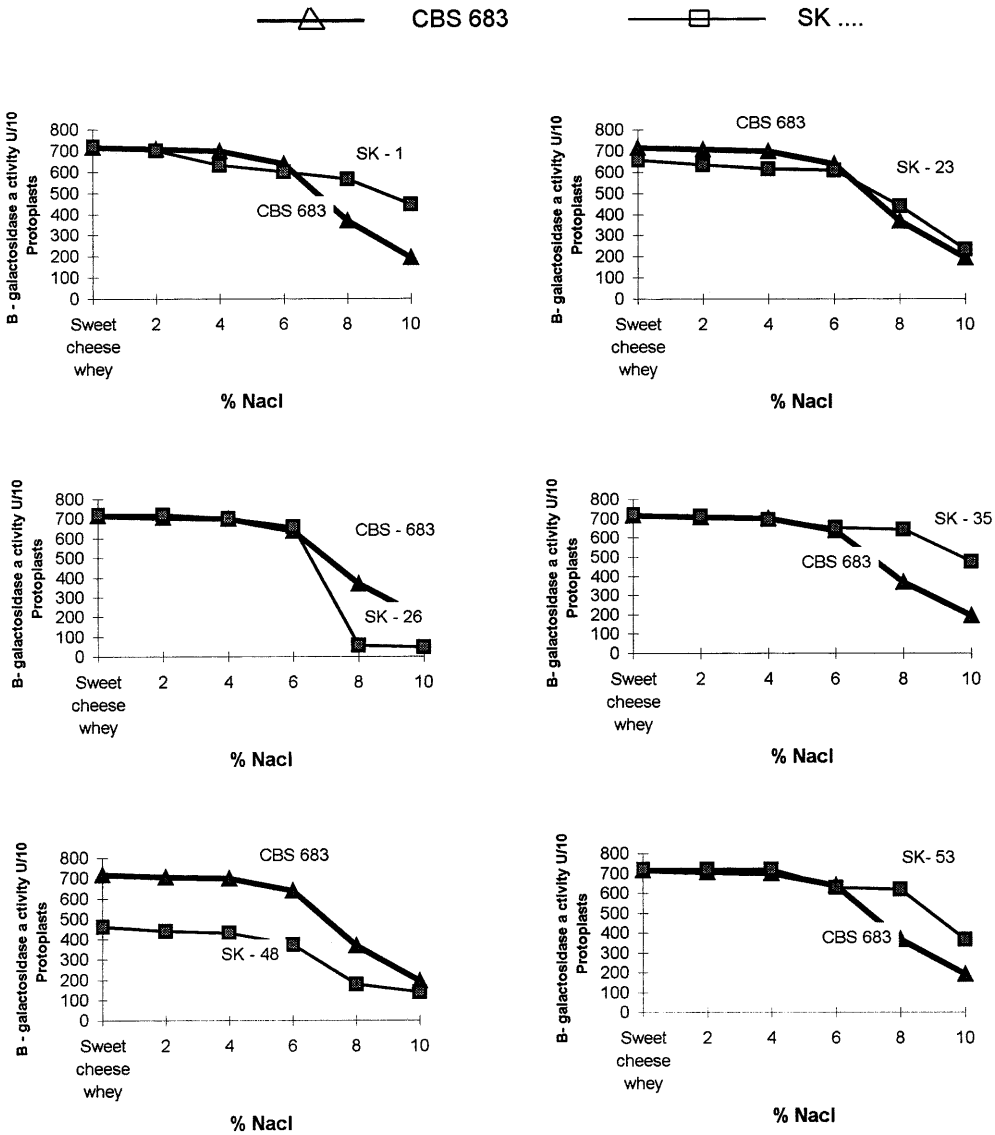

Fig. 1 β-Galactosidase activity units per 10^6 protoplasts by *Kluyveromyces lactis* CBS 683 and some recombinants grown in cheese whey medium containing different concentrations of sodium chloride at 30 °C for 96 h

measurements including nuclear diameter, DNA content and the ratio of DNA conten per hybrid from each of the two parental strains. Subsequent investigations including β-galactosidase activity, growth at different concentrations of sodium chloride, alcohol production, gene stability and fermentability of sweet and salted whey by new hybrids compared to parental yeast strains were performed.

The relationship between nuclear diameter, DNA content of recombinant per parent and ethanol yield by some representative recombinants showing high ethanol yield are shown in Table 1. The examined recombinants exhibited a higher DNA content of recombinant per parent and a higher nuclear diameter compared to the corresponding parental strains. This finding indicates that such recombinants are diploids ($2n$). Moreover, the new recombinants revealed higher ethanol yields that varied between 5.97 ml dl^{-1} (v/v) for recombinant strain SK-23 and 8.06 ml dl^{-1} (v/v) for recombinant strain SK-35.

The highest ethanol yield using the synthetic medium was 8.06% ml dl^{-1} (v/v) from 10 g dl^{-1} (w/v) lactose by recombinant SK-35. Such a value is greater than the values reported previously [20], which indicated an ethanol yield of 4.3 ml dl^{-1} (v/v) from 25 g dl^{-1} (w/v) lactose by a fusant strain PN13 that was obtained from the crossing of *S. cerevisiae* and *K. lactis.* Similarly, the ethanol yield obtained in the present study was higher than that reported by Spencer et al. [21] for a haploid recombinant *S. cerevisiae* strain that gave 2.07 ml dl^{-1} (v/v) ethanol from 10 g dl^{-1} (w/v) lactose. Woo et al. [8] had found an ethanol yield of 8.0 ml dl^{-1} (v/v) for recombinant fusant strain F-3-19 obtained from the intergeneric protoplast fusion of *S. cerevisiae* and *K. fragilis* CBS 397. On the other hand, Farahnak et al. [7] measured an ethanol yield of 13 ml dl^{-1} (v/v) for a recombinant strain that was prepared by intergeneric fusion of *S. cerevisiae* and *K. fragilis.*

In order to determine whether the recombinants obtained are the result of intraspecific fusion events of *S. cerevisiae* with *S. cerevisiae,* or *K. lactis* with *K. lactis,* or if they are the progeny of intergeneric fusion between the two strains, the fusant colonies were grown on YEPD medium containing 10 g dl^{-1} (w/v) sucrose. The data indicated that recombinants SK-1, SK-23, SK-26, SK-35 and SK-53 could grow on sucrose, whereas SK-48 did not reveal any growth. Accordingly, the latter recombinant is considered to be the result of the fusion event between two *K. lactis* nuclei. The other recombinants are considered as recombinant *S. cerevisiae* with a lactase system that has been transformed from *K. lactis.*

For utilization of available lactose in salted cheese whey, the examined recombinants were tested with respect to their efficiencies for growth on different concentrations of sodium chloride in a synthetic medium. The data revealed growth of the recombinants to different degrees on YEPD medium containing up to 10 g dl^{-1} (w/v) sodium chloride as compared to *S. cerevi-*

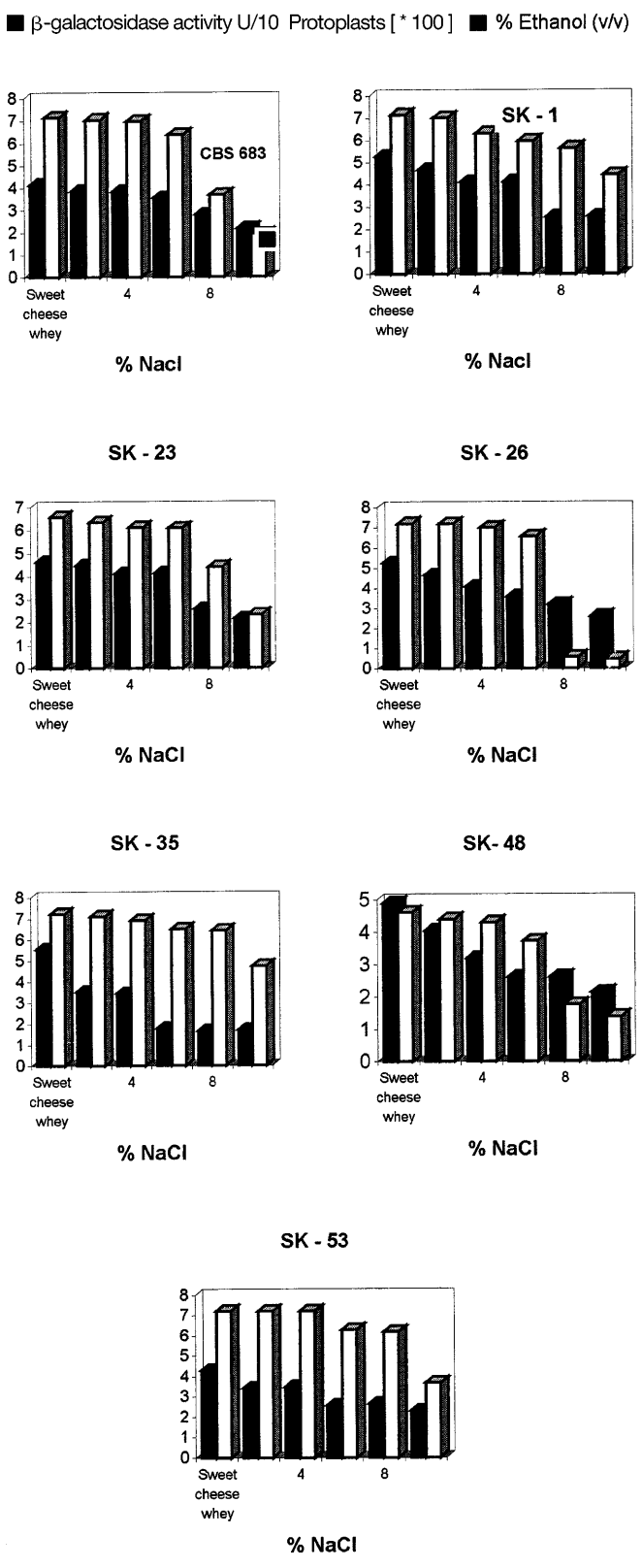

Fig. 2 Relationship between ethanol production and β-galactosidase activity by *K. lactis* CBS 683 and some recombinants grown in cheese whey medium containing different concentrations of sodium chloride at 30 °C for 96 h

Table 2 Ethanol yield (ml dl^{-1}) (v/v) by *K. lactis* CBS 683 and some recombinants grown in cheese whey medium containing different concentrations of sodium chloride at 30 °C for 96 h (average of four replicates)

Nuclei	Ethanol yield (ml dl^{-1}) (v/v) Sodium chloride (g dl^{-1}) (w/v)					
	0	2	4	6	8	10
CBS 683	4.15	3.85	3.83	3.55	2.86	2.16
SK-1	5.29	4.69	4.66	4.66	2.57	2.60
SK-23	4.65	4.48	4.13	4.14	2.59	2.17
SK-26	5.28	4.66	4.10	3.60	3.20	2.60
SK-35	5.55	3.53	3.45	1.77	1.65	1.71
SK-48	4.90	4.05	3.20	2.62	2.62	2.14
SK-53	4.30	3.43	3.50	2.58	2.66	2.13

siae, which exhibited vigorous growth in the presence of sodium chloride at concentrations of up to 10 g dl^{-1} (w/v).

The efficiency of a yeast strain to convert lactose to ethanol depends on the availability of an intracellular lactose system including β-galactosidase, galactoside permease and galactoside transacylase. The influence of the presence of sodium chloride in the growth medium on β-galactosidase activity and consequently on the rate of lactose utilization by parental *K. lactis* and the recombinants was investigated. Figure 1 indicates that raising the concentration of sodium chloride in cheese whey medium was accompanied by decreased β-galactosidase activity for parental as well as for recombinant strains. A similar tendency was detected for the relationship between the β-galactosidase activity and ethanol yield as shown in Fig. 2, indicating lower ethanol yields as a result of decreased enzyme activity. Table 2 illustrates the relationship between the concentration of sodium chloride in the cheese whey medium and ethanol yields of the recombinants and parental *K. lactis* CBS 683. The results indicate that raising the concentration of sodium chloride was accompanied by lower ethanol yields. However, some recombinants revealed higher tolerance to sodium chloride than parental *K. lactis.* Thus, recombinant strain SK-48 showed tolerance to 2 g dl^{-1} (w/v) sodium chloride, giving an ethanol yield of 4.05 ml dl^{-1} (v/v). Similarly, SK-1 exhibited tolerance up to 4 g dl^{-1} (w/v) sodium chloride, with an ethanol yield of 4.66 ml dl^{-1} (v/v). SK-23 could grow in 6 dl^{-1} (w/v) sodium chloride and give an ethanol yield of 4.14 ml dl^{-1} (v/v).

From the above results it can be concluded that strain SK-23 is as a suitable recombinant strain that can grow in 6 g dl^{-1} (w/v) sodium chloride and give an ethanol yield of 4.14 ml dl^{-1} (v/v) from cheese whey medium containing 4.6 g dl^{-1} (w/v) lactose.

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