Appl Microbiol Biotechnol (1999) 51: 621–626

ORIGINAL PAPER

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Construction of a flocculent *Saccharomyces cerevisiae* fermenting lactose

Received: 2 October 1998 / Received revision: 15 January 1999 / Accepted: 17 January 1999

Abstract A flocculent *Saccharomyces cerevisiae* strain with the ability to express both the *LAC4* (coding for β-galactosidase) and *LAC12* (coding for lactose permease) genes of *Kluyveromyces marxianus* was constructed. This recombinant strain is not only able to grow on lactose, but it can also ferment this substrate. To our knowledge this is the first time that a recombinant *S. cervisiae* has been found to ferment lactose in a way comparable to that of the existing lactose-fermenting yeast strains. Moreover, the flocculating capacity of the strain used in this work gives the process several advantages. On the one hand, it allows for operation in a continuous mode at high cell concentration, thus increasing the system's overall productivity; on the other hand, the biomass concentration in the effluent is reduced, thus decreasing product separation/purification costs.

Introduction

Cheese whey is a problem of great concern to the cheese industry because of its high biochemical oxygen demand content, ranging from 35 g/l to 60 g/l. Cheese whey is mainly composed of water, protein and lactose. The protein fraction can be removed by ultrafiltration and applied in the food industry while the lactose fraction, obtained as permeate (with a lactose concentration of 50–60 g/l), can be used for ethanol production by fermentation (Mawson 1987; Mann 1980). The success of this process depends upon the development of a high-productivity process for ethanol production. In order to increase productivity, techniques such as the use of high-cell-density systems have been thoroughly studied (Doran and Bailey 1986; Galazzo and Bailey 1990; Gikas and Livingston 1993; Kuriyama et al. 1993; Laplace et al. 1993). Among these, the use of flocculent microorganism in bioreactors, where their sedimentation characteristics can be exploited, is one of the most attractive. However, some of these processes, based on lactose-fermenting organisms are not always satisfactory in terms of yield and reliability. Not only is the number of microorganisms able to metabolise lactose directly to ethanol limited, but inhibition by moderate sugar and ethanol concentrations also occurs (Siso 1996). On the other hand, *Saccharomyces cerevisiae* is one of the organisms of first choice for industrial processes. Unfortunately this yeast lacks the lactose permease system as well as the intracellular enzyme for lactose hydrolysis, β-galactosidase, and thus is unable to ferment lactose directly into ethanol. Alternatives have been sought, such as the hydrolysis of lactose by β-galactosidase from another microorganism and subsequent fermentation by *S. cervisiae* (Champagne and Goulet 1988). However, when *S. cerevisiae* uses a mixture of glucose and galactose as a carbon source, diauxic growth occurs and lower yields of ethanol are obtained (Siso 1996). Other disadvantages of these processes are the high price of β-galactosidase and the failure of this enzyme to hydrolyse all the lactose, thus leaving problems associated with effluent disposal unsolved (Siso 1996).

Gene technology should make it possible to construct novel lactose-metabolising *S. cerevisiae* strains. Several attempts have been made in the past years to achieve the expression of the genes that code for β-galactosidase and the lactose permease system of *Kluyveromyces lactis* in *S. cerevisiae* (Russel 1986; Sreekrishna and Dickson 1985; Farahnak et al. 1986). However, the recombinant yeasts elaborated up to this point are very slow growing and have reduced genetic stability (Siso 1996), resulting in low yields even when these recombinant yeasts are used in specially designed bioreactors (Jeong et al. 1991). Alternative strategies involved the construction of *S. cerevisiae* cells that release β-galactosidase into the

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culture medium, either by secretion (Kumar et al. 1992) or as a result of overexpression of the transcriptional activator *GAL4*, which induced a partial lysis of the mother cells (Porro et al. 1992).

In this work, we report the construction of flocculent *S. cerevisiae* strains able to ferment lactose. A flocculent strain was used because the objective of this study is to obtain a high-productivity system for ethanol production from lactose.

Materials and methods

Strains and media

The bacterial strain used for DNA preparation was *Escherichia coli* HB101 grown in LB medium (1% casein, 0.5% yeast extract, 0.5% NaCl), according to Lennox (1955), containing 100 μg ampicillin/ml. LB plates were supplemented with 2% agar. The flocculent wild-type haploid strain of *S. cerevisiae* NCYC869 (*MATα FLO1*) was mutagenized by ultraviolet radiation, and orotidine-5′-phosphate-decarboxylase-deficient strains were isolated by positive selection using 5-fluoro-orotic acid as described previously (Boeke et al. 1984; Lima et al. 1995). The *ura3* mutation was confirmed by genetic complementation after transformation with the *URA3* gene (Venâncio et al. 1999). The auxotrophic strain A3 (*MATα FLO1 ura3*) was used for transformation experiments. For comparative studies, the yeast strain *Kluyveromyces marxianus* ATCC10022 was used. Yeasts were grown in complete YEPG medium (2% glucose; 2% peptone; 1% yeast extract) or minimal YNB medium (2% carbon source: glucose, galactose or lactose; 0.67% Difco yeast nitrogen base without amino acids) at 30 °C. A3 auxotrophic mutants were grown in YNB glucose medium with the appropriate supplement (50 μg/ml uracil). The yeast plates were supplemented with 2% agar.

Plasmids

Plasmid pYAC4 was purchased from Sigma (V-0758, lot 103H6770). This shuttle vector contains pBR322 + *CEN4* + *TEL* + *ARS1* + *TRP1* + *URA3* + *HIS3* (Burke et al. 1987). In the linearized form the *HIS3* gene is removed.

Plasmid pKR1B-Lac4-1 was purchased from ATCC (ATCC 40186, US patent 5, 047, 340 dated 10 September 1991). This vector is a derivative of pBR322 carrying the kanamycin-resistance gene (*G418*) of Tn903 and *ARS1B* from *K. lactis*. It also contains the genes *LAC4* and *LAC12* cloned from *K. lactis* Y1140, which code for β-galactosidase and for lactose permease respectively (Sreekrishna and Dickson 1985).

General DNA methods

Standard recombinant DNA methods were used (Maniatis et al. 1982). Enzymes were purchased from different manufacturers and were used as recommended. *E. coli* was transformed by electroporation according to protocols from Bio-Rad. DNA plasmid isolation from the recombinant yeast strain was done according to Hoffman and Winston (1987).

Yeast transformation

Yeast was transformed by the protoplast method according to Hinnen et al. (1978), or by the lithium acetate method of Ito et al. (1983), with the modifications referred to in Schiestl and Gietz (1989).

Transformant stability determination

Aliquots (500 μl) were successively withdrawn from the YEPG tube cultures to new tubes every 24 h over 5 days. The cells of the last aliquot were plated for single colonies onto YEPG and selective lactose/minimal-medium/agar plates, after being washed twice with 1.5% NaCl solution, pH 3.0, to ensure floc dispersion. The ratio of the number of colony-forming units in the selective agar plates (YNB/lactose) to those on the non-selective plates was used to provide the percentage of cells keeping the Lac^+ phenotype.

β-Galactosidase activity assays

Screening for β-galactosidase activity in yeast clones

The presence of β-galactosidase activity on the yeast clones was tested by a microtitre plate assay with *p*-nitrophenyl-β-D-galactopyranoside (pNp-Gal) as previously described (Domingues et al. 1997). A 100-μl sample of cell cultures was plated in a well and to each well 200 μl SDE buffer (0.05 M TRIS/HCl pH 7.5–8.0, 0.01 M EDTA, 1 M KCl; 0.05 M 2-mercaptoetanol), a drop of 0.1% (w/v) sodium dodecyl sulfate and a drop of chloroform were added. The β-galactosidase activity was detected by adding 50 μl 4 mg/ml pNp-Gal to each well, incubating the plates at 30 °C for 30 min and detecting positive wells by their yellow colour.

β-Galactosidase specific activity

The β-galactosidase specific activity was assayed in cellular crude extracts and determined in a total volume of 1.0 ml by following the hydrolysis of 2.3 mM pNp-Gal in buffer Z (Miller 1972), pH 7.0, spectrophotometrically at 420 nm. One unit of enzyme hydrolyses 1.0 nmol pNp-Gal/min at 25 °C. The value of the absorption coefficient of pNp-Gal was determined to be 6.6 mM^{-1} cm^{-1}. Soluble protein concentrations were assayed using Coomassie brilliant blue according to Bradford (1976).

Flocculation assay

Yeast cells were grown until stationary phase. The cells were washed twice with 1.5% NaCl, pH 3.0, solution. A 24-ml volume of cell suspension was placed on a 25-ml graduated cylinder. Then 1-ml 100 mM $CaCl_2$, pH 3.0, solution was added and the solution was immediately mixed by inversion 18 times. Samples of 200 μl were taken at the 20-ml level at different times. The sample absorbance was read at 620 nm (Soares et al. 1992). The control assay was made without addition of $CaCl_2$. The normalised cell concentration, defined as the ratio between actual and initial cell concentration, was plotted against sedimentation time. A sedimentation profile was then obtained.

Cell mass estimation

The free cell concentration was measured by dry weight and/or absorbance methods. The dry-weight cell concentration was determined by filtering the sample through 0.2-μm filter-paper and then drying it at 105 °C for 24 h. The absorbance was measured at 620 nm. A standard curve of absorbance against biomass concentration was previously constructed. Samples were treated with a deflocculation solution (NaCl 1.5%, pH 3.0) before the absorbance was read.

Lactose and ethanol measurement

Lactose concentration was determined by the dinitrosalicylic acid method (Miller 1959). Ethanol concentration was determined by gas chromatography with a Porapack Q column, flane ionisation detection, and isopropanol as internal standard.

Results

Construction of *S. cerevisiae* strains that grow on lactose

After unsuccessful attempts to transform the wild-strain *S. cerevisiae* NCYC869 with the plasmid pKR1B-Lac4-1, with either the lithium acetate method or the protoplast method, we undertook a co-transformation procedure for the uracil-deficient (*ura3*) strain *S. cerevisiae* NCYC869-A3. We chose to transform the co-transformation mixture directly into yeast as it has already been shown to be efficient in situations where direct selection for the desired product is possible (Gietz and Schiestl 1991). Therefore the co-transformation mixture (pKR1B-Lac4-1 + linear YAC4) was cloned into *S. cerevisiae* NYCY869-A3. With this methodology, *S. cerevisiae* cells growing on lactose were obtained. The selective medium used was 5-bromo-4-chloro-3-indolyl β-D-galactoside (Xgal)/YNB/galactose. In this medium, only the clones that have the *URA3* gene incorporated are able to grow. On the other hand, clones with the *LAC4* gene could be identified by the Xgal blue/white colour screen (Gietz and Sugino 1988). This approach enables the selection of Ura^+ clones and the indirect selection for positive Lac^+ transformants. The need for indirect selection has also been noted by Sreekrishna and Dickson (1985). From a total of 1212 transformants only 4 clones had the blue colour indicative of β-galactosidase. From these 4 clones, only 2, named T1 and T2, kept a stable Lac^+ phenotype. The transformants were thereafter kept in YNB/lactose medium, that is, imposing a double selection pressure.

Some modifications of the cell morphology of transformant T2 were observed. Both transformants presented some cells that were bigger than the traditional *S. cerevisiae* cell; the T2 transformant also presented bizarre forms and pseudo-mycelium (Fig. 1).

β-Galactosidase activity

Both transformants, T1 and T2, gave a positive colour reaction on screening for β-galactosidase and therefore the specific β-galactosidase activity was determined. In Table 1 the specific β-galactosidase activity for the different strains is presented. *S. cerevisiae* NCYC869 strain was used as control. Both *K. marxianus* and the transformed *S. cerevisiae* strains gave a positive reaction at the end of the assay.

Flocculation capacity

In earlier studies it was observed that the ability of the host strain *S. cerevisiae* NCYC869-A3 to flocculate is not affected by the lithium acetate transformation procedure (Venâncio et al. 1999). Even though the recombinant strains, T1 and T2, are able to flocculate they show different behaviour from the host strain. While the host strain maintains its flocculation capacity under moderate agitation speeds, the recombinant strains show a significant decrease in flocculation capacity even at 150 rpm (Fig. 2).

Growth on lactose

The growth on lactose was tested in YNB/lactose medium at different lactose concentrations. Surprisingly, the recombinant strains metabolised the same amount of

Fig. 1 Morphology of the recombinant T2 yeast cells. Magnification 500×

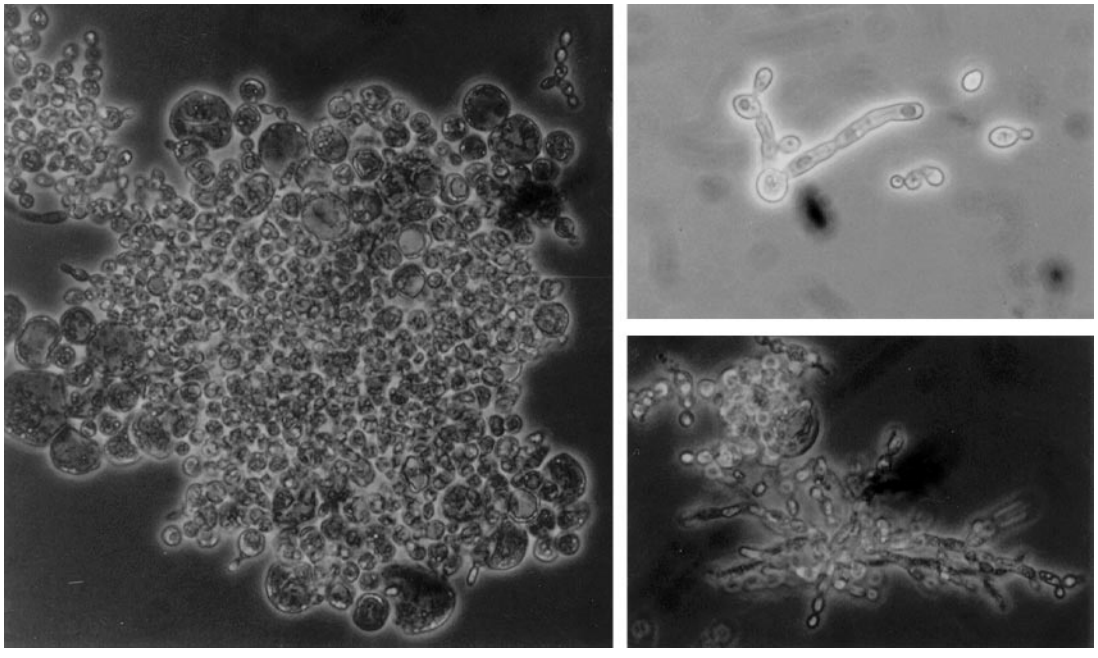

Table 1 Specific β-galactosidase activity for the recombinant strains T1 and T2, and for *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* strains. Results are means of at least three independent assays ± standard deviation

Strain	Specific β-galactosidase activity (U/mg protein)
Kluyveromyces marxianus	2360 ± 301
Saccharomyces cerevisiae	0
T1	343 ± 200
T2	138 ± 12

lactose (10 g/l) regardless of the initial lactose concentration (Fig. 3).

Nevertheless, β-galactosidase activity was present in the cells even when the lactose consumption stopped. Another intriguing aspect was that ethanol production was found to be residual and thus the biomass yields were close to those typical for oxidative metabolism. At this point the duplication time of the recombinant strain in lactose minimal medium was 5 h, which was better then the time reported by Sreekrishna and Dickson (6.7 h), but still slow when compared with the wild-type strains.

After an adaptation period, where the strain was kept in liquid lactose medium, refreshed periodically, the recombinant strain T1 was able to metabolise nearly 90 g/l lactose. Simultaneously, an increase in both the biomass-specific growth rate and the ethanol-specific production rate in lactose was observed. It was also observed that this adaptation period was needed even for an already adapted strain that had been kept in permanent culture at −80 °C. When the culture was regrown, the same limitation on lactose consumption, 10 g/l, was observed regardless of the initial lactose concentration.

In contrast, for the recombinant T2 the adaptation period had no effect on lactose consumption. Owing to these limitations on lactose consumption and on the cell morphology presented by the recombinant T2, only the transformant T1 was used for further studies.

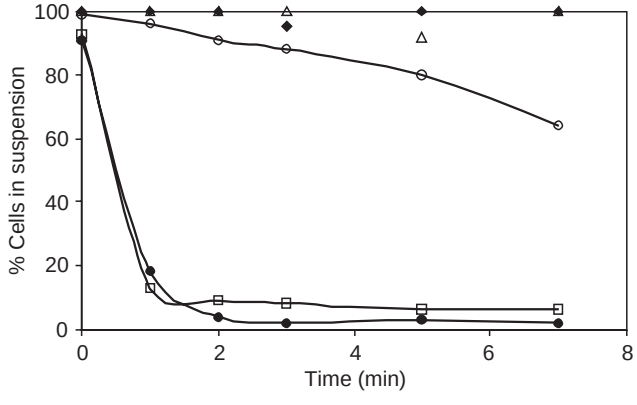

Fig. 2 Sedimentation profiles: △ *Kluyveromyces marxianus*, ◆ *Saccharomyces cerevisiae* NCYC869 without $CaCl_2$ (control), □ *Saccharomyces cerevisiae* NCYC869, ● recombinant T1 grown in static culture, ○ recombinant T1 grown at 150 rpm

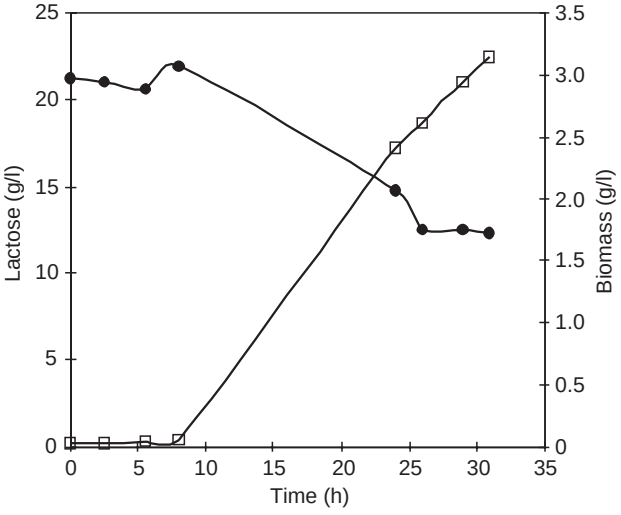

Fig. 3 Lactose (●) and biomass (□) concentration profiles for the recombinant T1, before the adaptation period in selective lactose minimal medium (20 g/l)

After the adaptation period, the recombinant T1 strain showed very good growth parameters on lactose and production of ethanol, as shown in Table 2. The effect of other nutrients rather then lactose was studied, doubling the concentration of YNB (2YNB) or quadrupling (4YNB), while keeping the lactose concentration constant.

Location of pKR1B-Lac4-1

After restriction digestion analysis, it could be observed that plasmids isolated from *S. cerevisiae* T1 and re-cloned in *E. coli* were identical to the KR1B-Lac4-1 plasmid (Fig. 4).

Transformant stability

After growing in non-selective conditions in YEPG medium, the stability of the Lac^+ phenotype for T1 strain was found to be 21% ± 3% and for T2 18% ± 2%.

Discussion

In this study, a genetically engineered strain of flocculent *S. cerevisiae* that expresses two *K. lactis* genes, an intracellular β-galactosidase and a most likely membrane-bound lactose permease, was successfully constructed. Previous results have been reported describing the transformation of a non-flocculent *S. cerevisiae* with plasmid KR1B-Lac4-1 (Sreekrishna and Dickson 1985). However, the growth rate of the recombinant strain was very slow and no results on ethanol production were presented.

After an adaptation period, our recombinant strain is not only able to grow in a medium with lactose as the

Table 2 Growth and ethanol production parameters on selective minimal medium for the recombinant strain T1, after the adaptation period, for different initial lactose concentrations (5–100 g/l). $Y_{X/S} = (S_f - S_0)/(X_f - X_0)$ (1); $Y_{P/S} = P_f/(S_f - S_0)$ (2); $\eta\,(\%) = Y_{P/S}/0.538 \times 100$ (3). *YNB, 2YNB, 4YNB* growth medium and double or four times concentrated medium

Parameter	5 g/l Lac			20 g/l Lac			50 g/l Lac			100 g/l Lac		
	YNB	2YNB	4YNB	YNB	2YNB	4YNB	YNB	2YNB	4YNB	YNB	2YNB	4YNB
Initial lactose concentration, S_0 (g/l)	5.3	5.2	5.1	18	18	19	48	48	43	92	94	89
Final lactose concentration, S_f (g/l)	0.1	0.1	0.1	0.1	0.1	0.2	0.7	0.2	0.2	30	4.6	2.5
Final biomass concentration, X_f (g/l)	1.0	1.1	1.1	1.8	1.8	1.9	2.8	3.1	3.2	3.2	4.3	4.8
Final ethanol concentration, P_f (g/l)	2.0	1.6	1.6	9.0	9.4	9.1	23.2	26.0	23.0	31.0	34.7	41.4
Substrate consumption yield, $Y_{X/S}$ (g/g)	0.19	0.21	0.21	0.10	0.10	0.10	0.06	0.06	0.07	0.06	0.05	0.05
Ethanol production yield, $Y_{P/S}$ (g/g)	0.33	0.24	0.26	0.47	0.50	0.45	0.48	0.53	0.53	0.50	0.38	0.47
Ethanol conversion yield, η (%)	61	44	48	88	94	84	89	99	98	92	71	88

sole carbon source but it can also ferment this substrate, thus presenting very good fermentation parameters on lactose. As can be observed in Table 2, the concentration of limiting nutrients other than lactose only had an effect above a lactose concentration of 50 g/l. At an initial lactose concentration above 90 g/l the duplication of other nutrients allowed an increase in the lactose consumption from 62 g/l to 89 g/l (44%). This highlights the case where lactose is no longer the limiting substrate. When the lactose concentration is increased an increase in the ethanol production yield from lactose and a decrease in the biomass conversion yield is observed. This is in agreement with the respiratory bottleneck model of *S. cerevisiae* (Sonnleitner and Käppeli 1986). This model is based on the fact that glucose degradation proceeds via two pathways under conditions of aerobic ethanol formation. It is considered that there is a limited respiratory capacity of *Saccharomyces*-type yeasts. Until there is a certain amount of substrate flux, the substrate is metabolised purely oxidatively. If substrate flux is beyond the respiratory capacity of the cells, the residual glucose is metabolised reductively and ethanol is produced. In the present case, although the substrate utilised is lactose, this sugar is hydrolysed into glucose and galactose inside the cells, and therefore it should not come as a surprise that the bottleneck model holds.

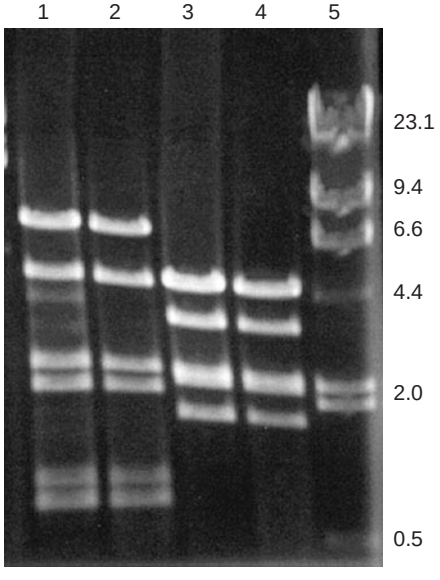

Fig. 4 Agarose gel electrophoresis of digested pKR1B-Lac4-1 and the plasmid isolated from the recombinant yeast T1. Lanes: *1*, *3* pKR1B-Lac4-1 used for yeast transformation, digested with *Eco*RI and *Pst*I respectively; *2*, *4* plasmid isolated from *S. cerevisiae* and recloned in *Escherichia coli* HB101 digested with *Eco*RI and *Pst*I respectively; *5* size marker (*Hind*III-digested λ DNA) shown in kb

The stability of the Lac^+ phenotype, after growth in non-selective conditions in YEPG medium, is in agreement with that obtained by Sreekrishna and Dickson (1985) for the KR1B-Lac4-1 plasmid. However, these authors suggest that the Lac^+ phenotype arises only when pKR1B-Lac4-1 integrates into one or a few specific chromosomal locations, and that this low stability is due to deletion of most of the *K. lactis* sequences from the integrated vectors. In this study, the low stability is most probably due to loss of plasmid under non-selective conditions, as plasmid KR1B-Lac4-1 has been isolated from the recombinant strain (Fig. 4).

The main goal of this work was to obtain a good lactose-fermenting *S. cerevisiae* strain that is flocculent. This raises new perspectives for ethanol production from lactose-composed raw materials. Even though the flocculation capacity of the recombinant strain showed some instability, this could be overcome by a selective bio-reactor operation for the flocculent cells (Mota and Teixeira 1990; Teixeira et al. 1990). Further work will be focused on continuous high-cell-density operation with this recombinant *S. cerevisiae* strain.

Acknowledgements The authors acknowledge the financial support provided by the Instituto de Biotecnologia e Química Fina (IBQF), Portugal. Lucília Domingues was supported by a grant Praxis XXI/BD/11306/97 from FCT (Fundação para a Ciência e Tecnologia), Portugal.

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