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Lactose fermentation by recombinant *Saccharomyces cerevisiae* strains

Pedro M. R. Guimarães, José A. Teixeira, Lucília Domingues*
IBB – Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710–057 Braga, Portugal.

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Abstract

The development of *Saccharomyces cerevisiae* strains with the ability to ferment lactose has a high biotechnological interest, particularly for cheese whey bioremediation processes with simultaneous bio-ethanol production. We have developed a flocculent *S. cerevisiae* strain that efficiently ferments lactose to ethanol, using a combination of genetic engineering and evolutionary engineering approaches. This strain fermented efficiently and nearly completely (residual lactose < 3 $g{\cdot}L^{-1}$) lactose concentrations up to 150 $g{\cdot}L^{-1}$, including 3-fold concentrated cheese whey, producing ethanol titres up to 8% (v/v). The ethanol productivity obtained with this strain (> 1.5 $g{\cdot}L^{-1}{\cdot}h^{-1}$) was higher than that reported for batch or fed-batch fermentations with other lactose-consuming recombinant *S. cerevisiae* strains. The strain is highly flocculent, a property that makes it interesting for the development of high cell density fermentation processes, which may attain much higher productivity.

Introduction

Cheese whey is a by-product of dairy industries, particularly the watery portion that is formed during the coagulation of milk casein in cheese making or in casein manufacture. Whey is produced in large amounts (about 9 litres of whey are generated per each Kg of cheese produced) and has a high polluting load, therefore creating a significant environmental problem (Siso, 1996). The world whey production is estimated to be around 82 million tons per year (Pesta et al., 2007). In Portugal, the production of liquid whey is estimated to be 500 – 560 thousand tons per year (Frazão, 2001), and the largest part of it is processed by concentration and drying. Whey has a BOD (biochemical oxygen demand) of 30 – 50 $g{\cdot}L^{-1}$ and a COD (chemical oxygen demand) of 60 – 80 $g{\cdot}L^{-1}$. Lactose is largely responsible for these high BOD and COD; protein recovery reduces the COD only by about 10 $g{\cdot}L^{-1}$ (Siso, 1996; Domingues et al., 1999a) On the opposite hand, however, whey represents about 85 – 95% of the milk volume and retains 55% of milk nutrients. Among the most abundant of these nutrients are lactose (4.5 – 5% w/v), soluble proteins (0.6 – 0.8% w/v), lipids (0.4 – 0.5% w/v) and mineral salts (8 – 10% of dried extract) (Siso, 1996). Therefore, whey has a vast potential as a source of added value compounds, challenging the dairy industry to face whey surplus as a resource and not solely as a waste problem.

The first step in most procedures for cheese whey valorisation consists in the recovery of the protein fraction. Whey proteins have high nutritional value as well as reported health benefits. Separation of whey proteins is typically done by ultrafiltration, producing whey protein concentrates (WPC), as well as high volumes of a lactose-rich stream, the permeate, that remains a major pollutant since it retains the lactose. The lactose in the permeate (ca. 5% w/v) may be used as a substrate for the production of valuable compounds by fermentation. The classical examples are ethanol and single cell protein (SCP) production in yeast-based

* Corresponding author. Tel + 351-253-604402. E-mail: luciliad@deb.uminho.pt

bioprocesses, although biotechnologists have proposed a multitude of alternative bio-products (see e.g. Siso, 1996; Pesta et al., 2007).

Thus, a bioprocess for the production of ethanol from the lactose in cheese whey can perform two roles: on the one hand it can reduce the whey polluting load, contributing to cheese whey bioremediation, and, on the other hand, an useful product (ethanol) is obtained that can be used as fuel or in food-related applications (e.g. in vinegar manufacturing or in the beverage industry). Being a waste product, whey has advantage over food-related fermentation feedstocks, such as corn, for bio-ethanol production.

Direct fermentation of whey or whey permeate to ethanol is hardly economically competitive with the currently established processes (using cane sugar and cornstarch as substrates) or with emerging second generation technologies (using lignocellulosic biomass as raw material), mainly because the low lactose content (ca. 5% w/v) results in low ethanol titre (2 – 3% v/v), making the distillation process too expensive. Concentration of whey lactose, e.g. by ultrafiltration, is an option to obtain higher ethanol titres. The use of cheese whey powder as an alternative source of concentrated lactose to the production of ethanol has also been recently proposed (Kargi and Ozmihci, 2006; Ozmihci and Kargi, 2007). High ethanol concentrations (10 – 12% v/v) may be obtained by fermentation of concentrated lactose media (up to 200 g L^{-1} lactose) thus significantly reducing the distillation costs. Microbial strains are needed that can efficiently convert such high concentrations of lactose into ethanol, allowing the development of fermentation processes that reach high ethanol titres as well as high ethanol productivities. Such processes must be designed to minimise residual lactose at the end of fermentation, since one of the major motivations for whey utilization is to reduce/eliminate its polluting load.

There are a few established industrial systems to produce ethanol from whey, which has been done in some countries, such as Ireland, Denmark, United States and New Zealand (Siso, 1996; Pesta et al., 2007). Recently, a German dairy company (Müllermilch) announced the construction of a plant near Dresden to produce 10 million liters of bio-ethanol per year from dairy by-products.

Kluyveromyces fragilis has been the microorganism of choice for most industrial plants producing ethanol from whey (Siso, 1996; Pesta et al., 2007), because *Saccharomyces cerevisiae*, the yeast usually chosen for industrial processes involving alcoholic fermentation, is unable to metabolise lactose. Nevertheless, the ability to metabolise lactose can be transferred to *S. cerevisiae* strains by genetic engineering, a problem that has been addressed over the past 20 years by several strategies (for a review see e.g. Rubio-Texeira, 2006). One of those strategies involves cloning of the *LAC4* (β-galactosidase) and *LAC12* (lactose permease) genes of the lactose-consuming yeast *Kluyveromyces lactis* in *S. cerevisiae* strains (Sreekrishna and Dickson, 1985; Rubio-Texeira et al., 1998). However, most strains obtained displayed undesirable characteristics (such as slow growth, genetic instability or problems derived from the use of glucose-galactose mixtures) or were ineffective for ethanol production.

This paper provides an outline of our recent work on engineering a flocculent *S. cerevisiae* strain for efficient lactose to ethanol fermentation. Detailed accounts of the work described here have already been published elsewhere (Guimarães et al., 2008a; Guimarães et al., 2008b).

Construction of a flocculent lactose-consuming *S. cerevisiae* strain

A recombinant *S. cerevisiae* strain with the ability to express both the *LAC4* and *LAC12* genes of *K. lactis* was initially constructed (for details see Domingues et al., 1999b). The plasmid KR1B-LAC4-1 (Sreekrishna and Dickson, 1985), which harbors the *LAC4* and *LAC12* genes, was co-transformed with a linear fragment from the plasmid YAC4 containing the *URA3* gene. The host strain (NCYC869-A3) was a uracil-deficient mutant of the flocculent haploid *S. cerevisiae* strain NCYC869 (*MATα FLO1*).

Using this strategy, a recombinant strain able to metabolize lactose was obtained. However, that original recombinant (strain T1) showed a rather poor lactose fermentation phenotype, growing slowly in lactose and presenting a low ethanol conversion yield (Table 1). The flocculation ability of the strain T1 was also poor when compared with the host strain (Guimarães et al., 2008a).

Evolutionary engineering of the recombinant strain for efficient lactose to ethanol fermentation

In order to improve the lactose fermentation phenotype of the recombinant strain (T1) we used an evolutionary engineering approach. Evolutionary engineering refers to the exploitation of the evolutionary principles to enhance microbial properties in a biotechnological context, provided the desired phenotype is amenable to direct or indirect selection (Sauer, 2001). Evolutionary engineering approaches have been used successfully in the improvement of genetically engineered *S. cerevisiae* strains for xylose and arabinose fermentation (see e.g. Sonderegger and Sauer, 2003; Wisselink et al., 2007).

The strategy that we used to improve the lactose fermentation performance of the recombinant strain T1 consisted in serial transfer/dilution in gently shaken (40 rpm) flasks (for details see Guimarães et al., 2008a). This strategy was designed to keep the recombinant growing in lactose for many generations (>120), as well as to select for the most flocculent cells. The cells recovered at the end of the experiment presented significantly improved lactose fermentation performance compared to T1. These evolved cells were considered to be an independent strain, which was named T1-E (evolved T1). The evolved recombinant strain, T1-E, consumed lactose 2-fold faster and produced 30% more ethanol than T1 (Table 1). Moreover, T1-E flocculated in an earlier phase of the fermentations and formed much bigger flocs than T1, as could be easily observed by visual inspection of the cultivation flasks.

Table 1 – Comparison of fermentation parameters of strains T1 and T1-E in shake-flask cultivations with 25 $g \cdot L^{-1}$ of lactose. Data from a similar fermentation with a *K. lactis* strain (CBS2359) are also show for comparison.[1]

Yeast strain	T1	T1-E	*K. lactis*
Specific growth rate (h^{-1})	0.14 ± 0.01	0.21 ± 0.01	0.28
Final biomass concentration ($g \cdot L^{-1}$)	3.48 ± 0.09	2.81 ± 0.09	2.56
Maximum ethanol concentration ($g \cdot L^{-1}$)	7.08 ± 0.79	10.52 ± 0.04	8.86
Ethanol conversion yield (% of theoretical)	53 ± 5	69 ± 1	65

[1]Shake-flask fermentations were done at 30 ºC with 150 rpm agitation in yeast defined mineral medium (for details see Guimarães et al., 2008a). Data are means ± ranges of duplicate independent cultivations for T1, and means ± standard deviations of triplicate cultivations with single colony isolates for T1-E. Data for *K. lactis* are from a single cultivation.

A series of physiological and genetic studies were done to compare the original recombinant and the evolved strain. Two molecular events that targeted the *LAC* construct in the evolved strain were identified: (1) a 1593 bp deletion in the intergenic region (promoter) between *LAC4* and *LAC12*, and (2) a decrease of the plasmid copy number by about 10-fold compared to the original recombinant. The construction of T1 involved the transfer of a 13 kb *K. lactis* genomic sequence that included the genes *LAC4* and *LAC12* as well as their intergenic region (LACIR) (Domingues et al., 1999b). LACIR is an unusually large intergenic region that works as a promoter for the divergent transcription of both the *LAC* genes. This region contains four functional upstream activating sites (UASs) that synergistically contribute to the activation of both genes by providing binding sites for the transcriptional activator Lac9p, homologous to Gal4p of *S. cerevisiae* (Gödecke et al., 1991). Our results suggest that the intact promoter (endogenous *K. lactis* promoter) was unable to mediate induction by

lactose of the transcription of *LAC4* and *LAC12* in the original recombinant T1, whereas the deletion identified established transcriptional induction of both genes in the evolved recombinant T1-E. We proposed that tuning of the expression of the heterologous *LAC* genes in the evolved recombinant was accomplished by interplay between the decreased copy number of both genes and different levels of transcriptional induction for *LAC4* and *LAC12*, resulting from the changed promoter structure (Guimarães et al., 2008a).

Fermentation of high concentrations of lactose by the evolved recombinant strain

The performance of the evolved recombinant strain (T1-E) was studied in batch fermentations with high concentrations of lactose (up to 200 $g \cdot L^{-1}$). Since aeration has been reported to improve the performance of yeast in fermentations producing very high concentrations of ethanol (see e.g. Alfenore et al., 2004) we have tested T1-E in micro-aerated (shake-flasks) and in well-aerated (bubble column bioreactor) fermentations.

The fermentations were performed in defined mineral medium. Shake-flask fermentations were done in Erlenmeyer flasks filled with medium to 40% of the total volume and incubated at 30 ºC and 150 rpm. The initial pH was adjusted to 5.5 and 100 mM potassium hydrogen phthalate was added to the medium to avoid major drops in pH during cultivation. The bioreactor fermentations were done in a 600 mL bubble column (made in-house) filled with 440 mL of medium. The temperature was maintained at 30 ºC and the pH at 4.0. An air flow rate of 1 vvm was applied using a sintered porous plate located at the bottom of the column and a magnetic stirrer was used to aid in keeping the entire volume well-mixed (for details about the fermentations see Guimarães et al., 2008b).

The evolved recombinant strain consumed rapidly and completely 150 $g \cdot L^{-1}$ lactose in either well-aerated or micro-aerated batch fermentations, producing 5.5% (v/v) and 8.0% (v/v) of ethanol, respectively (Table 2). However, the yeast was unable to consume completely 200 $g \cdot L^{-1}$ lactose in the micro-aerated shake-flask fermentations, but, conversely, consumed totally the same concentration of lactose in well-aerated bioreactor fermentations, although taking a considerable time (about 60 h) to complete fermentation. This may be related with the yeast's inability to synthesize proper amounts of sterols and unsaturated fatty acids under the oxygen limiting conditions found in the shake-flask fermentations. It is well established that yeast needs oxygen in order to synthesise unsaturated fatty acids and sterols (see e.g. Guimarães et al., 2006), and lower amounts of these lipids in the plasma membrane have been correlated with lower ethanol tolerance (see Aguilera et al., 2006 and references therein).

Table 2 – Fermentation of high concentrations of lactose by T1-E in micro-aerated (shake-flasks) and in aerated (bubble column bioreactor) cultivations

Micro-aerated				Aerated			
Initial lactose ($g \cdot L^{-1}$)	Ethanol produced (% v/v)	Ethanol productivity ($g \cdot L^{-1} \cdot h^{-1}$)	Residual lactose ($g \cdot L^{-1}$)	Initial lactose ($g \cdot L^{-1}$)	Ethanol produced (% v/v)	Ethanol productivity ($g \cdot L^{-1} \cdot h^{-1}$)	Residual lactose ($g \cdot L^{-1}$)
114	6.1	2.0	< 1.5	100	4.1	1.2	< 1.5
150	8.0	1.5	< 1.5	150	5.5	1.6	< 1.5
200	8.4	1.4	50	200	7.2	0.95	< 1.5

The ethanol yields were much higher in the shake-flask fermentations (about 80% of the theoretical) than in the bioreactor fermentations (< 60%). This probably resulted from lower oxygen availability for yeast in the shake-flasks (conditions of micro-aeration), leading to

higher sugar flux towards fermentative metabolism. Therefore, under our conditions high aeration was not beneficial to the ethanol production performance of the yeast.

In the design of processes for ethanol production from cheese whey or whey permeate a compromise must be made between maximisation of ethanol titre and productivity and minimisation of the residual lactose concentration at the end of fermentation, since one of the major motivations for whey utilization is to reduce its polluting load. The results suggest that an initial lactose concentration of 150 $g \cdot L^{-1}$ represents the best compromise to obtain high ethanol titre (8% v/v) and productivity (1.5 $g \cdot L^{-1} \cdot h^{-1}$) at the end of fermentation (i.e. with a lactose residual < 1.5 $g \cdot L^{-1}$).

The ethanol productivity obtained in this work was higher than that reported for batch or fed-batch fermentations with other lactose-consuming recombinant *S. cerevisiae* strains: 0.3 $g \cdot L^{-1} \cdot h^{-1}$ (Rubio-Texeira et al., 1998); 0.14 to 0.6 $g \cdot L^{-1} \cdot h^{-1}$ (Ramakrishnan and Hartley, 1993); 1 $g \cdot L^{-1} \cdot h^{-1}$ (Compagno et al., 1995); 1.3 $g \cdot L^{-1} \cdot h^{-1}$ (Farahnak et al., 1986). The utilization of high initial lactose concentrations enabled also to attain higher ethanol titres than previously obtained with recombinant *S. cerevisiae*, with the exception of the work of (Farahnak et al., 1986) that reported an ethanol titre of 13% (v/v). The strain used here was also able to ferment concentrated cheese whey powder solution containing about 150 $g \cdot L^{-1}$ lactose in a batch fermentation with low (0.1 vvm) aeration, consuming nearly all lactose (residual lactose < 3 $g \cdot L^{-1}$) in about 120 h and producing 7% (v/v) ethanol, which corresponds to an ethanol productivity of 0.46 $g \cdot L^{-1} \cdot h^{-1}$ (Guimarães et al., 2008a). The performance of the yeast may be improved by supplementation of the cheese whey with cheap nutrient sources (e.g. corn steep liquor). To our knowledge, the recombinant strain used in this work is the most efficient lactose-fermenting *S. cerevisiae* strain reported in the literature. An additional important advantage of this strain is its highly flocculent phenotype, which makes it particularly suitable for the development of high cell density fermentation processes that, when operated in continuous with flocculated biomass retention, may allow to attain very high ethanol productivities (Domingues et al., 2000).

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