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ALCOHOLIC FERMENTATION OF LACTOSE BY ENGINEERED FLOCCULENT *SACCHAROMYCES CEREVISIAE*

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ABSTRACT
The construction of *Saccharomyces cerevisiae* strains with the ability to ferment lactose has biotechnological interest, particularly for cheese whey fermentation to ethanol. Direct fermentation of whey to ethanol is generally not economically feasible 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) prior to fermentation is an option to obtain higher ethanol titres. Microbial strains are therefore needed that can efficiently convert high concentrations of lactose into ethanol. We describe here the engineering of a *S. cerevisiae* strain for efficient lactose fermentation, involving genetic and evolutionary engineering strategies. The evolved strain obtained fermented efficiently lactose concentrations up to 150 $g \cdot L^{-1}$, including 3-fold concentrated cheese whey, producing ethanol titres up to 8% v/v. The strain is highly flocculent, a property that makes it particularly suitable for the development of high cell density fermentation processes.

Keywords: Lactose fermentation; ethanol production; cheese whey; recombinant *S. cerevisiae*; flocculent yeast; evolutionary engineering.

INTRODUCTION
Cheese whey is a by-product of the 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 and has a high polluting charge therefore creating a significant environmental problem [1]. On the other 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). Whey also contains appreciable quantities of other components, such as lactic (0.05% w/v) and citric acids, non-protein nitrogen compounds (urea and uric acid) and B group vitamins [1]. Therefore, whey has a vast potential as a source of added value compounds, challenging the industry to face whey surplus as a resource and not only as a waste problem.
Cheese whey represents an important environmental problem because of the high volumes produced and its high organic matter content. To make 1 kg of cheese, about 9 litres of whey are generated [2]. The world whey production is estimated to be about 82 million tons per year [3]. In Portugal, the production of liquid whey is estimated to be 500 – 560 thousand tons per year [4], and the largest part of it is processed by concentration and drying [5]. Whey exhibits a BOD (biochemical oxygen demand) of 30 – 50 g/L and a COD (chemical oxygen demand) of 60 – 80 g/L. Lactose is largely responsible for the high BOD and COD. Protein recovery reduces the COD of whey only by about 10 g/L [1, 6].
The first step in most procedures for cheese whey valorisation consists in the recovery of the protein fraction. Whey proteins represent about 20% of the milk proteins, having a high nutritional

value [1] as well as reported health benefits [7]. The most abundant proteins in whey are β-lactoglobulin (55 – 65%), α-lactalbumin (15 – 25%), immunoglobulins (10 – 15%), bovine serum albumin (5 – 10%) and lactoferrin (1 – 2%) [7]. Separation of whey proteins is typically achieved by ultrafiltration to produce whey protein concentrates (WPC) [1, 2]. High volumes of a lactose-rich stream, the permeate, are also obtained during this process. The permeate remains a major pollutant since it retains the lactose. Lactose in permeate (ca. 5%) 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 bioprocesses, although biotechnologists have proposed a multitude of alternative bio-products [1, 3, 8]. Large whey surplus together with the need for cheap and largely available substrates and, above all, the rapid advances in microbial biotechnology are likely to prompt further exploitation of whey lactose as fermentation feedstock to obtain value-added products.

Being a waste product, whey has advantage over food-related fermentation feedstocks, such as corn, for bio-ethanol production. In addition, lactose fermentation strongly reduces the whey polluting charge, contributing to solve the environmental problem created by whey surplus. Moreover, whey ethanol has food-grade quality, and therefore can find a proper market e.g. in vinegar manufacturing and in the beverage industry. 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 [9, 10]. High ethanol concentrations (10 – 12% v/v) may be obtained by fermentation of concentrated lactose solutions (up to 200 $g \cdot L^{-1}$ lactose) thus reducing 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 charge [8].

There are a few established industrial systems to produce ethanol from whey [3], which has been done in some countries, such as Ireland, Denmark, United States and New Zealand [1, 3, 11]. A German dairy company (Müllermilch) is building 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 [1], though other lactose-fermenting yeasts (*Kluyveromyces marxianus* or *Candida pseudotropicalis*) have also been considered [3]. On the other hand, *Saccharomyces cerevisiae* is usually the first choice for industrial processes involving alcoholic fermentation, mainly because of its good fermentative capacity, ethanol tolerance, capacity to grow under anaerobic conditions and extensive industrial use. However, this yeast is unable to metabolise lactose. The engineering of *S. cerevisiae* for lactose utilization has been addressed over the past 20 years by different strategies [12]. 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, as is the case of *S. cerevisiae* strains expressing the *LAC4* (β-galactosidase) and *LAC12* (lactose permease) genes of *Kluyveromyces lactis* [13-15], with the exception of the strain described here.

This paper provides an outline of our recent work on engineering a *S. cerevisiae* strain for efficient lactose fermentation, as well as fermentation of high concentrations of lactose (up to 200 $g \cdot L^{-1}$) to ethanol by that same strain. The strain is highly flocculent, a property that makes it particularly suitable for the development of high cell density fermentation processes [16].

Detailed accounts of the work outlined here have already been published [17] or have been submitted for publication in peer-reviewed scientific journals.

ENGINEERING *S. CEREVISIAE* FOR EFFICIENT FERMENTATION OF LACTOSE

Our initial approach involved the use of “classical” genetic engineering to transfer the *LAC* genes of *Kluyveromyces lactis* to a flocculent *S. cerevisiae* strain (NCYC869). Thus, a lactose-consuming *S.*

cerevisiae recombinant expressing the *LAC12* (lactose permease) and *LAC4* (β-galactosidase) genes of *Kluyveromyces lactis* was obtained (for details see [18]). This original recombinant (strain T1) was able to grown on lactose, but its lactose fermentation performance was rather poor (Table 1). Moreover, the flocculation performance of the recombinant was poor when compared to the host strain *S. cerevisiae* NCYC869-A3 (uracil-deficient mutant of strain NCYC869) [18]

In order to improve the lactose fermentation phenotype of the strain T1 we followed a simple evolutionary engineering strategy. 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 [19]. Evolutionary engineering of whole cells is gaining relevance both as a complementary strategy in metabolic engineering for strain development and as a tool to elucidate the molecular basis of desired phenotypes. A strain with specific properties obtained by rational metabolic engineering can be subjected to evolutionary engineering for further improvement, as illustrated by our work. Likewise, evolutionary engineering can generate a strain with desirable properties that can then be further optimised by rational metabolic design, thus integrating and complementing classical metabolic engineering strategies [20].

Our approach to improve the lactose fermentation performance of the original recombinant T1 consisted in a serial transfer/dilution strategy in gently shaken (40 rpm) flasks (for details see [17]). This strategy was designed to keep the recombinant growing in lactose for many generations (>120), as well as to select for flocculent cells. Therefore, in some stages of the process, the medium was simply refreshed periodically in the same cultivation flask: the cultivation broth was decanted and fresh medium was added to the flocculated cells, which had sedimented to the bottom of the flask. The yeast cells recovered at the end of the process 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). The experiment was also successful in the selection of cells with improved flocculation: T1-E flocculated earlier and formed much bigger flocs than T1, as could be easily observed by visual inspection of the cultivation flasks.

Table 1 – Comparison of the fermentation parameters of strains T1 and T1-E in shake-flask cultivations with 25 g/L of lactose. Data from a similar fermentation with *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 defined mineral medium [21]. The concentrations of trace elements and vitamins in the medium were doubled. The medium was supplemented with100 mM potassium hydrogen phthalate to avoid major drops in the pH during cultivation (initial pH adjusted to 4.5; final pH > 3.7 in all fermentations). 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.

We undertook a series of physiological and genetic studies 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) [18]. 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* [22]. 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 [17].

FERMENTATION OF HIGH CONCENTRATIONS OF LACTOSE WITH THE EVOLVED *S. CEREVISIAE* RECOMBINANT STRAIN T1-E

The evolved recombinant strain, T1-E, was tested in shake-flask fermentations with mineral medium [21] with initial lactose concentrations of 100 – 200 $g \cdot L^{-1}$ (Table 2).

The yeast completely consumed 114 $g \cdot L^{-1}$ initial lactose in about 24 h, producing 48 $g \cdot L^{-1}$ ethanol. This corresponds to an ethanol productivity of 2 $g \cdot L^{-1} \cdot h^{-1}$ and an ethanol conversion yield 78% of the theoretical.

In fermentations with about 150 $g \cdot L^{-1}$ initial lactose concentration, T1-E produced 55 - 59 $g \cdot L^{-1}$ ethanol in about 28 h. At this point, ethanol productivity was highest (2 $g \cdot L^{-1} \cdot h^{-1}$). However, there was still a lactose residual of 17 - 34 $g \cdot L^{-1}$, which was further consumed attaining a final residual of < 1.5 $g \cdot L^{-1}$ lactose after about 45 h. At 40 - 45 h, ethanol concentration was highest (ca. 63 $g \cdot L^{-1}$) but ethanol productivity had decreased to 1.5 $g \cdot L^{-1} \cdot h^{-1}$. The ethanol conversion yield was 78 - 84% of the theoretical yield.

With 200 $g \cdot L^{-1}$ initial lactose, the ethanol productivity was maximal (1.7 $g \cdot L^{-1} \cdot h^{-1}$) at 27 h, corresponding to an ethanol concentration of 46 $g \cdot L^{-1}$ and a lactose residual of 95 $g \cdot L^{-1}$. Ethanol concentration was highest (66 $g \cdot L^{-1}$) after 48 h of fermentation, corresponding to a productivity of 1.4 $g \cdot L^{-1} \cdot h^{-1}$. A lactose residual of about 50 $g \cdot L^{-1}$ remained unconsumed. Yeast growth was only slightly slower with 200 $g \cdot L^{-1}$ initial lactose than that with 150 $g \cdot L^{-1}$ initial lactose.

Table 2 – Fermentation of high concentrations of lactose by T1-E.[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
150	8.0	1.5	< 1.5
200	8.4	1.4	50

[1]Fermentations were done in shake-flasks (30 ºC, 150 rpm) with defined mineral medium [21]. The concentrations of trace elements and vitamins were doubled. The medium was supplemented with 100 mM potassium hydrogen phthalate to avoid major drops in the pH during cultivation (initial pH adjusted to 5.5; final pH > 4.3 in all fermentations).

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 in the effluent, since the purpose of the process is also waste treatment. The recombinant *S. cerevisiae* strain T1-E consumed rapidly and completely 150 $g \cdot L^{-1}$ of lactose in micro-aerated batch fermentations (shake-flasks). The yeast was however unable to totally consume 200 $g \cdot L^{-1}$ of lactose in these fermentations. The yeast growth and ethanol production (maximum ethanol titre of ca. 8% v/v) were similar with either 150 or 200 $g \cdot L^{-1}$ initial lactose concentration. The highest ethanol productivity was 2 $g \cdot L^{-1} \cdot h^{-1}$ with 150 $g \cdot L^{-1}$ initial lactose. However, when the fermentation reached completion (i.e. with a lactose residual < 1.5 $g \cdot L^{-1}$ and a maximal ethanol titre of 8% v/v) ethanol productivity was considerably lower (1.5 $g \cdot L^{-1} \cdot h^{-1}$).

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 $g \cdot L^{-1} \cdot h^{-1}$ [14]; 0.14 to 0.6 $g \cdot L^{-1} \cdot h^{-1}$ [23]; 1 $g \cdot L^{-1} \cdot h^{-1}$ [24]; 1.3 $g \cdot L^{-1} \cdot h^{-1}$ [25]. 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.* [25] that reported an ethanol titre of 13% (v/v).

The evolved strain was also able to ferment concentrated cheese whey powder solution containing about 150 $g \cdot L^{-1}$ lactose in a bioreactor 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 55 $g \cdot L^{-1}$ of ethanol, which corresponds to an ethanol productivity of 0.46 $g \cdot L^{-1} \cdot h^{-1}$ [17]. To our knowledge, the recombinant strain T1-E is the most efficient lactose-fermenting *S. cerevisiae* strain reported in the literature, providing an attractive alternative for the fermentation of lactose-based media.

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