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Engineered autolytic yeast strains secreting *Kluyveromyces lactis* β-galactosidase for production of heterologous proteins in lactose media

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Abstract

Secretion of the heterologous *Kluyveromyces lactis* β-galactosidase into culture medium by several *Saccharomyces cerevisiae* osmotic-remedial thermosensitive-autolytic mutants was assayed and proved that new metabolic abilities were conferred since the constructed strains were able to grow in lactose-containing media. Cell growth became independent of a lactose-uptake mechanism. Higher levels of extra-cellular and intra-cellular β-galactosidase production, lactose consumption and growth were obtained with the LHDP1 strain, showing a thermosensitive-autolytic phenotype as well as being peptidase-defective. The recombinant strain LHDP1 presented the highest β-galactosidase yields from biomass and the lowest ethanol levels from lactose. This strain is effective for the heterologous production and release of *K. lactis* β-galactosidase into the extra-cellular medium after osmotic shock.

Keywords: β-Galactosidase; Protein secretion; Milk-whey; Autolytic yeast strains

1. Introduction

Lactose is the sugar present in milk and is therefore important from a nutritional and biotechnological point of view. Lactose is the main solid constituent of whey, a byproduct of cheese production whose disposal represents a serious environmental concern because of its high biological oxygen demand (González-Siso, 1996). Direct utilization of this sugar is difficult because its sweetening power and solubility are low and there are only a few microorganisms able to utilize lactose as their only source of carbon and energy. Industrial applications of processes based on the enzymatic hydrolysis of lactose of milk-whey with β-galactosidase or lactase (E.C. 3.2.1.32) increase the value of milk-whey as a food product and as a microbial substrate. However, the industrial development of such processes has been limited because of the high cost associated with extraction and

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downstream processing due to the intra-cellular nature of the yeast enzyme and to the low yields obtained due to enzymatic instability.

The construction of *Saccharomyces cerevisiae* strains able to efficiently metabolize lactose and, therefore, to grow in the waste effluent cheese-whey, constitutes an important biotechnological objective. The first attempt to ferment cheese-whey by yeast were based on the enzymatic pre-hydrolysis of lactose but *S. cerevisiae* showed diauxic growth in the mixture of glucose and galactose and these processes had limited applicability (Porro et al., 1992a). Afterwards, genetic engineering techniques allowed the heterologous expression in *S. cerevisiae* of the enzymatic system necessary for lactose degradation. Earlier developed recombinant strains were genetically unstable and showed very slow growth rates (Sreekrishna and Dickson, 1985; Vanoni et al., 1989; Porro et al., 1992b). Both characteristics improved for some of the most recently developed strains that express the β-galactosidase and lactose permease genes from *Kluyveromyces lactis*, performing an intra-cellular hydrolysis of the disaccharide (Rubio-Texeira et al., 1998, 2001; Adam et al., 1999; Domingues et al., 1999a,b). In this work an alternative approach, which consists of the construction of *S. cerevisiae* osmotic-remedial thermosensitive-autolytic mutants in which the *K. lactis* β-galactosidase is secreted, has been assayed, thus avoiding the need for a permease and allowing faster lactose hydrolysis. The main distinctive feature of the transformed strains is the thermosensitive-autolytic phenotype. These strains lyse at non-permissive temperatures due to mutations altering cell wall formation (Álvarez et al., 1994) and are useful in the release, following an osmotic shock, of heterologous *K. lactis* β-galactosidase (Becerra et al., 1997). The release by osmotic shock constitutes an easy and efficient way to obtain high yields of *K. lactis* β-galactosidase in the extra-cellular medium without any mechanical breakage, chemical extraction or enzymatic treatment of the cells, thereby contributing to a reduction in the production costs of this enzyme.

The objective of this work was to test the ability of these recombinant yeast strains to grow on lactose media. The significance of the developed strains is two-fold: they offer a profitable use for milk-whey and they afford a cheap alternative to intra-cellular protein production thereby reducing downstream processing costs.

2. Materials and methods

2.1. Strains and plasmids

The following *S. cerevisiae* strains were used: LD1 (*MATa/MATα lyt2-1/lyt2-1 leu2-3.112/leu2-3.112 his4Δ34/his4Δ34*) and LHDP1 (*MATa lyt2-1 ade2 leu2-3.112 pep4::HIS3 prb1Δ1.6R*) (Álvarez et al., 1994). The autolytic LD1 and LHDP1 strains were provided by Drs. C. Nombela and M. Molina from Universidad Complutense (Madrid, Spain). The mutant strain PEP4-1a (*MATα leu2 ura3 pep4-3*) was used as a control. PEP4-1a is a protease-deficient strain obtained from Dr. C.P. Hollenberg and his colleagues at Heinrich-Heine Universität (Düsseldorf, Germany).

Yeast strains were transformed using the lithium acetate procedure (Ito et al., 1983). Plasmid uptake and β-galactosidase production by the transformed strains were identified on plates with the chromogenic substrate X-gal in the corresponding auxotrophic medium.

LD1, LHDP1 and PEP4-1a strains were transformed with plasmid YEplac181-LAC4 (Becerra et al., 2001a) bearing the *LAC4* gene, which codes for *K. lactis* β-galactosidase, fused in frame to the secretion signal of the *K. lactis* killer toxin and expressed under the control of the promoter and terminator of the phosphoglycerate-kinase (*PGK*) gene from *S. cerevisiae*.

2.2. Media and culture conditions

Liquid batch cultures of transformed strains were grown in Erlenmeyer flasks filled with 20% volume of culture medium stirred at 250 rpm, unless otherwise stated.

YPD (1% yeast extract, 2% bactopeptone, 2% dextrose), YPL (1% yeast extract, 2% bactopeptone, 4 or 6% lactose) and cheese-whey (ultrafiltration permeate) were used as culture media. The ultrafiltration permeate of cheese-whey (about 5% lactose) was obtained from a local dairy plant (Quegalsa, Ferrol, A Coruña, Spain). If a protein precipitate was observed

after autoclave sterilization of permeate at 121 °C for 15 min, it was removed by centrifugation (15 min at 8000 × *g*).

As inocula, a suitable volume of a stationary phase preculture in complex medium (Zitomer and Hall, 1976) without the corresponding auxotrophic amino acid was added to obtain an initial OD_{600} of 0.05. Samples were taken at regular time intervals to measure growth (OD_{600}), intra- and extra-cellular β-galactosidase activity, lactose consumption, and ethanol production.

2.3. Osmotic shock

The recombinant strains were incubated at 37 °C and 250 rpm in 100 ml of cheese-whey ultrafiltration permeate containing 1 M sorbitol. The thermosensitive lytic phenotype caused by *lyt2-1* is compensated by osmotic stabilization with sorbitol (Álvarez et al., 1995). After 77 h of culture, cells were precipitated by centrifugation and resuspended in an equal volume of 10 mM Tris–HCl, pH 7.5. Intra- and extra-cellular β-galactosidase activity was measured before and after the osmotic shock.

2.4. Plasmid stability

Samples were taken after several days of culture and 100 μl of a suitable dilution were spread on YPD plates supplemented with the chromogenic substrate X-gal. Cells containing the plasmid appeared blue when growing on this medium.

2.5. β-Galactosidase activity assays

The method of Guarente (1983) as previously described (Becerra et al., 2001b) was used. One enzyme unit (EU) was defined as the amount of enzyme that catalyzes the release of 1 μmol of *ortho*-nitrophenol from *ortho*-nitrophenyl-β-D-galactopyranoside per min under assay conditions. EU are expressed per ml of culture medium.

Throughout this paper and unless otherwise specified, the term extra-cellular β-galactosidase is used to mean the enzyme in the culture medium and the term intra-cellular β-galactosidase is used to mean the cell-associated enzyme, both periplasmic and cytoplasmic.

2.6. Lactose and ethanol determinations

Lactose and ethanol were determined using enzyme-based kits available from Boehringer-Mannheim (Germany) (Lactose kit: Cat. No. 176 303; Ethanol kit: Cat. No. 176 290).

3. Results and discussion

Two different osmotic-remedial *S. cerevisiae* mutants, which lyse at non-permissive temperature due to mutations altering cell wall formation, were used: LD1, a diploid strain homozygotic for the mutant allele *lyt2-1* that induces autolysis when cells grow at the non-permissive temperature 37 °C (Torres et al., 1991; Fuente et al., 1992), and LHDP1, a haploid strain with reduced protease activity due to *pep4* and *prb1* mutations which also contains the mutant allele *lyt2-1* (Jones et al., 1982). A protease-deficient strain, PEP4-1a, was used for comparative purposes and as a control. All strains were transformed with plasmid YEplac181-LAC4 (Becerra et al., 2001a) carrying the *LAC4* gene. Although generally for heterologous secretion of proteins the α-factor signal sequence from *S. cerevisiae* is used, in this work the secretion of the enzyme in these strains is directed from this episomal plasmid by the secretion signal of the *K. lactis* killer toxin under the control of the constitutive promoter and terminator of the phosphoglyerate-kinase gene from *S. cerevisiae*. Growth and intra- and extra-cellular β-galactosidase production by the recombinant strains were measured in glucose and lactose complete media.

3.1. Growth of the recombinant S. cerevisiae osmotic-remedial mutants in glucose media

Representative parameters of the flask batch cultures of the *S. cerevisiae* osmotic-remedial mutants transformed with YEplac181-LAC4 on YPD growing at 25 °C are shown in Fig. 1. The percentage of β-galactosidase secretion into the culture medium was higher in the first 48 h of culture (about 20 and 27% for LD1 and LHDP1, respectively, of total activity when intra-cellular activity was low) than after this time (2.33% for LD1 and 0.7% for LHDP1). Intra-cellular levels of β-galactosidase activity were an average of

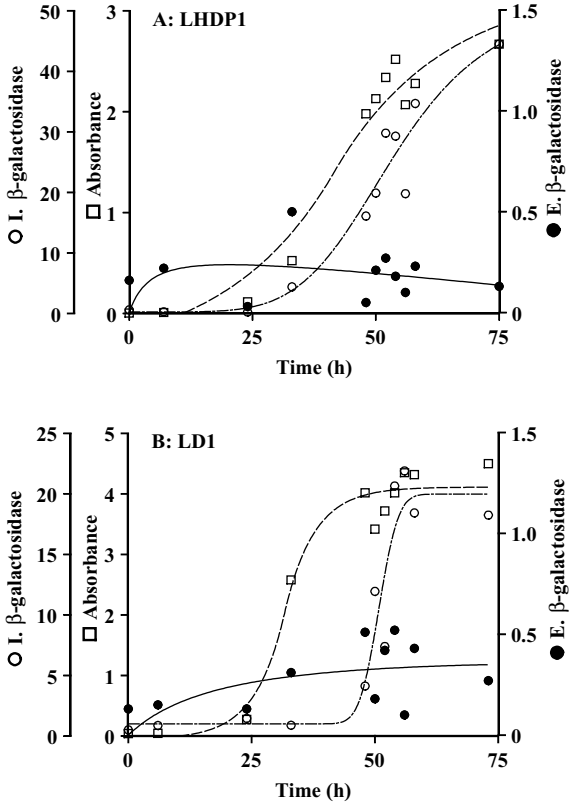

Fig. 1. Growth (absorbance at 600 nm) (squares and dashed line), extra-cellular (E) (filled circles and solid line) and intra-cellular (I) (open circles and dash-dotted line) β-galactosidase activity (EU ml^{-1}) by the transformed LHDP1 strain (A) and the LD1 strain (B) in YPD media. All errors are <10% of point value.

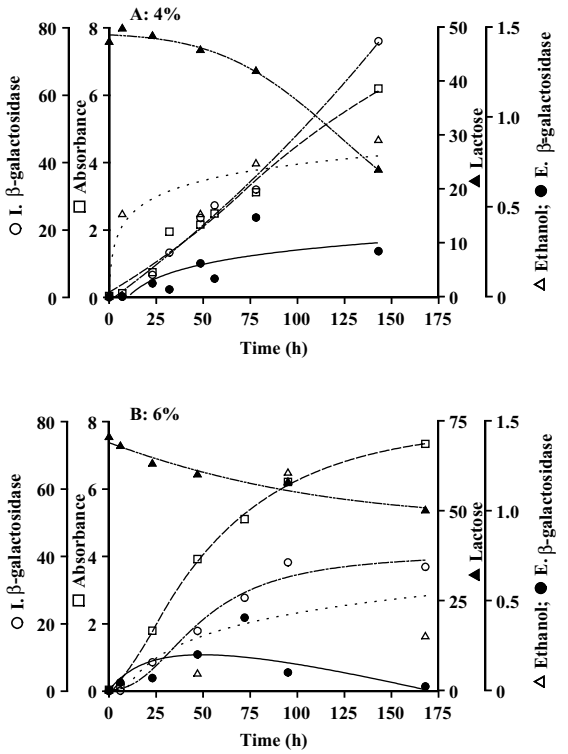

Fig. 2. Growth (absorbance at 600 nm) (squares and dashed line), extra-cellular (E) (filled circles and solid line) and intra-cellular (I) (open circles and dash-dotted line) β-galactosidase activity (EU ml^{-1}), lactose consumption (g l^{-1}) (filled triangles and dash-dot-dotted line) and ethanol production (g l^{-1}) (open triangles and dotted line) by the transformed LHDP1 strain in YPL-4% medium (A) and in YPL-6% medium (B). All errors are <10% of point value.

2.5 times higher in LHDP1 than in LD1, possibly attributed to a reduction in β-galactosidase degradation due to *pep4* and *prb1* mutations.

Plasmid stability during growth in YPD for the two transformed strains was determined after 72 h of culture. A stability of about 70 and 80% was obtained for the LHDP1 and LD1 strains, respectively.

3.2. Growth of the recombinant S. cerevisiae osmotic-remedial mutants in lactose media

Although the percentages of β-galactosidase secretion obtained with cultures growing in YPD were not too high, cultures of the recombinant strains were prepared in YPL media with two different amounts of lactose (4, 6%) to test the ability of these recombinant yeast strains to grow on lactose media. Both strains were able to grow on lactose as a carbon source (Figs. 2 and 3).

Although the non-permissive temperature to these mutant strains is 37 °C, the cultures were grown at 30 °C, instead of 25 °C as recommended to avoid lysis (Álvarez et al., 1994). The temperature chosen may produce a slight degree of cell lysis and favor β-galactosidase secretion into the culture medium. However, the lysis caused by growth at 30 °C of the two autolytic strains does not seem to be relevant. So, LHDP1 showed levels of extra-cellular β-galactosidase like those reached at 25 °C in YPD medium and LD1 produced reduced amounts of extra-cellular β-galactosidase (Fig. 3); the levels of

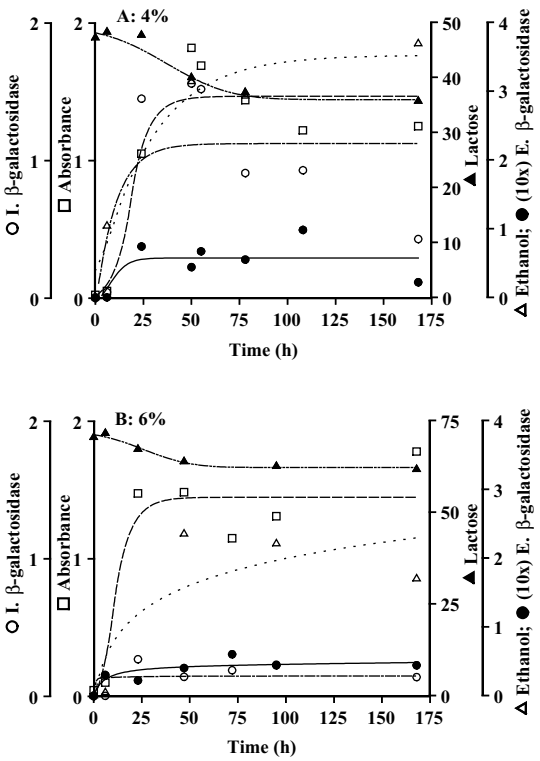

Fig. 3. Growth (absorbance at 600 nm) (squares and dashed line), extra-cellular (E) (filled circles and solid line) and intra-cellular (I) (open circles and dash-dotted line) β-galactosidase activity (EU ml^{-1}), lactose consumption (g l^{-1}) (filled triangles and dash-dot-dotted line) and ethanol production (g l^{-1}) (open triangles and dotted line) by the transformed LD1 strain in YPL-4% medium (A) and in YPL-6% medium (B). All errors are <10% of point value.

extra-cellular β-galactosidase for LD1 were around three times lower than in the case of LHDP1.

Lactose consumption was 23.7 g l^{-1} (at 143 h of culture) for the recombinant LHDP1 in 4% lactose YP-medium and 20.39 g l^{-1} (at 168 h of culture) in 6% lactose (Fig. 2). However, for the recombinant LD1, lactose consumption was at 168 h of culture 11.5 g l^{-1} (YPL-4%) and 8.7 g l^{-1} (YPL-6%) (Fig. 3).

Plasmid stability was, after 6 days of culture in YPL-4% medium, 71 and 78% for the LHDP1 and LD1 strains, respectively.

The intra-cellular levels of β-galactosidase for the LHDP1 strain were similar in the three media assayed in this work (YPD, YPL-4% and YPL-6%). As occurs in YPD media, intra-cellular levels of β-galactosidase activity were higher in LHDP1 than in LD1, probably because of a reduction in β-galactosidase degradation due to *pep4* and *prb1* mutations rather than to an increase in β-galactosidase synthesis. Intra-cellular accumulation of β-galactosidase was growth-associated as expected from a protein expressed under a constitutive promoter.

Estimated yields in β-galactosidase from biomass were higher in the case of the recombinant LHDP1 (12.3 EU mg^{-1} for YPL-4% and 5.03 EU mg^{-1} for YPL-6%) than in the other recombinant strains assayed in this work and were higher than those obtained by *K. lactis* in liquid- and solid-state cultures in deproteinized milk-whey (Becerra and González-Siso, 1996).

The autolytic strain LHDP1 yields higher OD_{600} (growth), lactose consumption and extra-cellular and intra-cellular β-galactosidase production than LD1. The better results obtained with the LHDP1 strain may be attributed to the fact that this strain is peptidase-defective, since both are *lyt2-1* mutants and were transformed with the same plasmid having similar stability range. Osmotic shock of the LHDP1 *S. cerevisiae* strain is useful for the release of heterologous intra-cellular high molecular weight proteins with good yields, as *K. lactis* β-galactosidase that reached 63% release into the medium after osmotic shock (Becerra et al., 1997). Use of the LHDP1 strain allows higher absolute levels of the protein to be obtained in the medium and also minimizes further proteolytic reactions due to the protease-deficient genotype.

The peptidase mutant strain, PEP4-1a, having a similar genotype to that of LHDP1 but without the *lyt2-1* mutation, transformed with the above referred plasmid, showed lower lactose consumption than the other two strains, being 3.3 g l^{-1} for the YPL-4% medium and 7.96 g l^{-1} for the YPL-6% medium. Extra-cellular β-galactosidase was around four times lower than that in the LHDP1 strain and 1.5 times lower than in the LD1 strain, and plasmid stability was 54% after 6 days of culture in YPL-4% medium.

Although not excluding the fact that lactose is also hydrolyzed in the periplasm where it is freely diffusible, a moderately strong correlation has been found between lactose utilization and extra-cellular β-galactosidase levels for the recombinant *Saccharomyces* using lactose reported here.

3.3. Ethanol production

Ethanol production was low in all cultures when the recombinant yeasts were grown on lactose (Figs. 2 and 3). With the exception of the LD1 strain, which reached 3.7 g ethanol l^{-1} in the 4%-lactose culture medium at 168 h of culture, the rest of the strains produced less than 1.5 g ethanol l^{-1}. Yields surpassed 50% of the theoretical maximum value (assuming that 4 mol of ethanol can be produced per mole of lactose used) in the cases of strains LD1 and PEP4-1a but were below 15% for LHDP1 even though shake flask cultures with high concentrations of sugar become rapidly oxygen-limited.

In addition to the biotechnological advantages, the development of *Saccharomyces* strains able to utilize lactose is interesting for the study of some aspects of metabolic regulation. The typical pattern of sugar utilization by *Saccharomyces* is fermentative, with occurrence of alcoholic fermentation under aerobic conditions (Crabtree effect; Pronk et al., 1996). The presence or absence of the Crabtree effect has biotechnological implications (Rubio-Texeira et al., 1998). A Crabtree-positive yeast directs a proportion of the glycolytic flux towards the formation of fermentation products (i.e. ethanol, acetate) whereas a yeast lacking this effect can utilize the sugar more energy-efficiently, which can be translated into higher biomass production.

The recombinant LHDP1 strain constructed in this work shows a predominantly oxidative lactose utilization as revealed by the low levels of ethanol present in the culture media. It exhibited a respiro-fermentative metabolism similar to that of *K. lactis*, with low ethanol production and high biomass yield. This behavior may be of interest in the transformation of lactose into biomass and biomass-associated products.

3.4. Growth of the recombinant S. cerevisiae osmotic-remedial mutants in cheese-whey

The ability of the recombinant strains to grow in ultrafiltration permeate of cheese-whey and to produce and release β-galactosidase into the extracellular medium after osmotic shock was assayed. The three recombinant strains were able to grow in this medium. As occurs in the other three media analyzed in this work, the LHDP1 strain showed the highest growth rate and intra-cellular and extra-cellular β-galactosidase production, β-galactosidase release into the medium reaching 51% after osmotic shock. Estimated yields in β-galactosidase from biomass were also higher in the case of the recombinant LHDP1 (around 5.78 EU mg^{-1}).

4. Conclusion

In this work, secretion of *K. lactis* β-galactosidase by several *S. cerevisiae* osmotic-remedial thermosensitive-autolytic mutants was assayed and proved that the constructed strains were able to grow in lactose-containing media. Cell growth became independent of a lactose-uptake mechanism. These strains could be of commercial interest since they allow the use of milk-whey, a byproduct whose disposal is expensive to many cheese industries. On the other hand, the autolytic phenotype is an interesting alternative to intra-cellular protein production and reduces downstream processing costs. The effectiveness of the recombinant LHDP1 strain for the heterologous production and release of β-galactosidase from *K. lactis* into the extra-cellular medium after osmotic shock, the high yield in β-galactosidase obtained from biomass and its capacity to grow in lactose-containing media makes this strain valuable to biotechnology and dairy industries.

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