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Acquisition of flocculation phenotype by *Kluyveromyces marxianus* when overexpressing *GAP1* gene encoding an isoform of glyceraldehyde-3-phosphate dehydrogenase

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Abstract

The use of flocculating yeast strains has been considered as a convenient approach to obtain high cell densities in bioreactors with increasing productivity in continuous operations. In *Kluyveromyces marxianus* ATTC 10022, the *GAP1* gene encodes an isoform of glyceraldehyde-3-phosphate dehydrogenase–p37—that is accumulated in the cell wall and is involved in flocculation. To test the use of p37 as a tool for engineering *Kluyveromyces* cells to display a flocculation phenotype, *K. marxianus* CCT 3172 was transformed with an expression vector containing *GAP1*. This vector is based on the pY37 previously described, harbouring a *S11 Kluyveromyces* origin of replication, and the expression of *GAP1* is under the control of *GAL1*. *Kluyveromyces* cells overexpressing *GAP1* acquired a flocculent phenotype together with the accumulation of p37 in the cell wall. The results support the use of *GAP1* gene as a molecular tool for inducing flocculation.
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1. Introduction

Yeasts are a growing source of enzymes, essentially used in the food processing industries (Walker, 1998). Compared with fungi cultures for protein production, yeast broths have a lower viscosity, together with a lower presence of contaminating species (e.g. proteases) simplifying both culture handling, and downstream separation. Furthermore, yeasts are particularly suitable for genetic manipulation and for heterologous gene expression and protein production (Walker, 1998). Lately, there has been an increasing interest in the use and study of non-conventional yeast strains, such as *Kluyveromyces*, *Pichia* and *Hansenula* spp.

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These strains have the advantages of growing on different substrates and to produce heterologous proteins with a higher level of secretion compared with *Saccharomyces cerevisiae* (Dominguez et al., 1998; Wésolowski-Louvel et al., 1996).

Using a bioreactor designed to work in continuous operation with a high cell density, enhances the target protein productivity and further simplify downstream separation. This high cell density bioreactors can be achieved by means of cell retention, which may involve entrapment in solid matrixes, attachment to small particles and fibres, or by imposing barriers to cell transport (Bailey and Ollis, 1986). Furthermore, it is possible to increase the cell concentration in the reactor by working with flocculating yeast strains, avoiding the use of expensive carriers and membranes (Domingues et al., 2000). To that end, it is relevant to develop a strategy that renders non-flocculating yeast into flocculating cells.

Cell flocculation can occur in few yeast strains and results from the non-sexual aggregation of single cells into multicellular masses, which then sediment from the medium (Stratford, 1994a,b,c).

The mechanisms underlying yeast flocculation have been extensively analysed namely for *S. cerevisiae* and it is clear that the cellular interaction is mediated by cell wall proteins (Miki et al., 1982; Stratford and Assinder, 1991). At present, in *S. cerevisiae* there are seven FLO genes associated with the flocculation phenotype, such a *FLO1*, *FLO5* or *FLO8* (Bidard et al., 1994; Stratford and Assinder, 1991; Stratford, 1994a,b,c; Teunissen et al., 1993a,b; Domingues et al., 2000).

Some *Kluyveromyces* spp. have also revealed a flocculation phenotype and that is well correlated with cell wall proteins (Géhin et al., 2001; Sousa et al., 1992; Teixeira et al., 1989). In *K. marxianus* ATCC 10022, it was revealed that an isoform of glyceraldehyde-3-phosphate dehydrogenase (p37) accumulates in the cell wall of flocculent cells. Furthermore, it was shown to be involved in the flocculation phenotype (Fernandes et al., 1992; Falcão-Moreira et al., 1998). The *GAP1* gene encoding the p37 has been characterized and is a member of a gene family with three genes (Fernandes et al., 1995).

The present work describes a strategy to convert a non-flocculent strain of *K. marxianus* to a flocculent strain by overexpressing the *GAP1* gene. Thus, this approach can be applied to *Kluyveromyces* endogenous enzymes production.

2. Materials and methods

2.1. Media and strains

The strains used in this work are listed in Table 1.

LB medium (1% (w/v) bacto-tryptone, 1% (w/v) sodium chloride and 0.5% (w/v) yeast extract) was used for bacteria growth.

The rich media (Y.P.) used for yeast growth included 2% (w/v) peptone, 1% (w/v) yeast extract and different concentrations of carbon source (glucose or galactose). The minimal media contained 0.67% (w/v) of yeast nitrogen base without amino acids (Difco) and different concentrations of carbon source (glucose or galactose).

2.2. Plasmids

To express *GAP1* gene in the non-flocculent strain *K. marxianus* CCT 3172, a *K. marxianus* expression vector was constructed. The plasmid was based on pY37, which is a *S. cerevisiae* expression vector containing the *URA3* genetic marker and the *K. marxianus GAP1* gene under the control of *GAL1* promoter (Falcão-Moreira et al., 2000). The plasmid pSK1 (Huo and Li, 1995) was used as template for the amplification of the *Kluyveromyces* origin *S11*. An empty vector derived from pYES2 (Invitrogen) and containing the *S11* origin was constructed and used as control (pKYES2).

Table 1
Strains used in this work *ura3* mutants from strain *K. marxianus* CCT 3172 were obtained using 5 fluoro-orotic-acid [19]

Strains	References
E. coli XL1 Blue	Stratagene
K. marxianus CCT 3172	supplied by A. Wheals
K. marxianus ATCC 10022	Teixeira et al., 1989
K. marxianus CCT 3172 *ura3*-	this work
K. marxianus CCT 3172 *ura3*-transformed with pKYES2	this work
K. marxianus CCT 3172 *ura3*-transformed with pKY37	this work

2.3. PCR

PCR was carried out using a mixture of 50 ng of plasmid pSK1, and PCR primers **A1** (5′ CGG CAC ATA CGT ACC CCG AAA AGT GCC 3′) and **A2** (5′ AAC TAC CGC TAC GTA AAA GCT TAT CGA TG 3′). The total volume of each reaction mixture was 50 μl and included 1 mM of each primer, Tris/HCl 100 mM pH 8.3, KCl 500 mM, $MgCl_2$ 15 mM, dNTPs 200 mM. The *Taq* DNA polymerase was included (1 U) after 10 min of denaturation at 94 °C. Thirty cycles of 94 °C for 1 min, 62 °C 1 min and 72 °C 2 min were followed by an extension step at 72 °C for 15 min. The PCR products were analysed and excised from a 0.8% agarose gel.

2.4. Bacterial transformation

E. coli competent cells were transformed following the $CaCl_2$ method (Ausubel et al., 1996). Transformed cells were grown overnight at 37 °C in LB solid medium with 50 μg ml^{-1} ampicillin.

2.5. Yeast transformation

K. marxianus ura3-competent cells were transformed by electroporation (Ausubel et al., 1996) and selected after 3 days of incubation at 26 °C in minimal medium (composition presented above) with 1 M sorbitol.

2.6. Electrophoresis and Western blotting

The proteins were analysed in SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) and transferred to nitrocellulose membranes (Amersham) by standard procedures. Protein p37 was detected using a rabbit anti-p37 serum (Fernandes et al., 1992).

2.7. Northern blotting

RNA was extracted from yeast cells using the hot acidic phenol method followed by electrophoresis in agarose gel (1.4%) and transfer to Hybond-N nylon membrane (Amersham-Pharmacia) (Ausubel et al., 1996). The hybridisation and detection procedures were performed using the AlkPhosDirect kit (Amersham-Pharmacia) according to the manufacturer instructions. *GAP1* ORF was used as probe.

2.8. Detection of biotinylated cell wall proteins

To biotinylate the yeast cell wall surface proteins, the method of Mrsa et al. (1997) was followed as described in Falcão-Moreira et al. (2000). SDS-soluble cell wall proteins were then extracted, separated in polyacrylamide gels and transferred to nitrocellulose membranes. To visualise total cell wall surface proteins, the membrane was incubated with an avidin reagent (streptavidin horseradish peroxidase), which binds biotin, and the proteins were detected by fluorescence (Mrsa et al., 1997).

2.9. Enzyme assays

Glyceraldehyde-3-phosphate dehydrogenase activity in the cytosol was assessed using the method described by Holland and Holland (1979).

2.10. Plasmid stability

A culture in YPD of *K. marxianus* CCT 3172 containing the pKY37 vector was grown in an orbital shaker at 120 rpm, 26 °C. After 24 h, 500 μl was withdrawn from the Erlenmeyer and served as the inoculum for fresh YPD medium. The process was repeated every 24 h for 3 days. At this time, the cells in 500 μl were spread in a Petri dish with YPD medium. After growth 48 h at 26 °C, 100 colonies were transferred to solid YNB + glucose medium and to solid YPD medium. The result was expressed comparing the number of colonies appearing in the minimal medium (corresponding to the cells containing the plasmid) to the ones grown in the control Petri dish (rich medium), as percent of total colonies in the control.

3. Results

3.1. Engineering of a vector for the expression of GAP1 gene in K. marxianus

To construct a vector for expression of genes under the control of the *GAL1* promoter and suitable for *K. marxianus*, a *S11* fragment (the origin of replication of

Table 2
Maximum specific growth rates (μ_{max}) for growth in YPD medium (WT—*K. marxianus* CCT 3172 wild type cells; pKYES2 and pKY37—*K. marxianus* CCT 3172 transformed with the vectors)

	WT	pKYES2	pKY37
μ_{max} (h^{-1})	0.552 ± 0.015	0.556 ± 0.004	0.560 ± 0.006
r^2	0.997	0.9998	0.9996

the plasmid pKD1 isolated from *K. drosophilarium* (Wésolowski-Louvel et al., 1996) was inserted into pYES2 and pY37.

The S11 origin of replication in *Kluyveromyces* was obtained by PCR using the pSK1 vector as template (Huo and Li, 1995). Both primers include a *Sna*BI restriction site leading to blunt ends. *S11* was then inserted in the unique restriction site (*Hpa*I) located inside the pYES2 . The new shuttle vector, which can be used for the expression of genes in *K. marxianus* under the control of *GAL1* promoter, was named pKYES2. This new vector has in the multiple cloning site several restriction sites, in contrast to other *Kluyveromyces* sp.

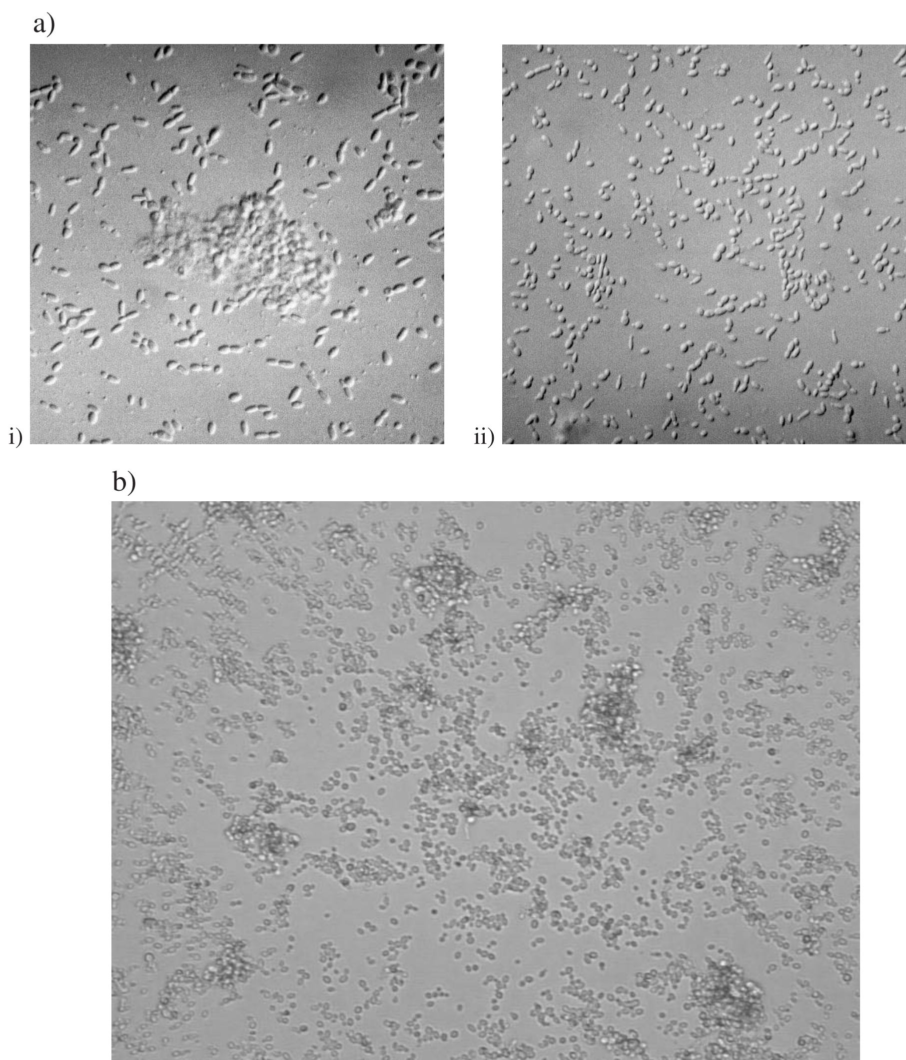

Fig. 1. (a) Aspect of a batch culture of the transformed cells with pKY37 (i) and with pKYES2 (ii) in YP + 2% galactose, 200 mM Ca^{2+} (magnified 400 ×). (b) Aspect of a culture of the transformed cell with pKY37 in 2% galactose, 200 mM Ca^{2+} (magnified 200 ×).

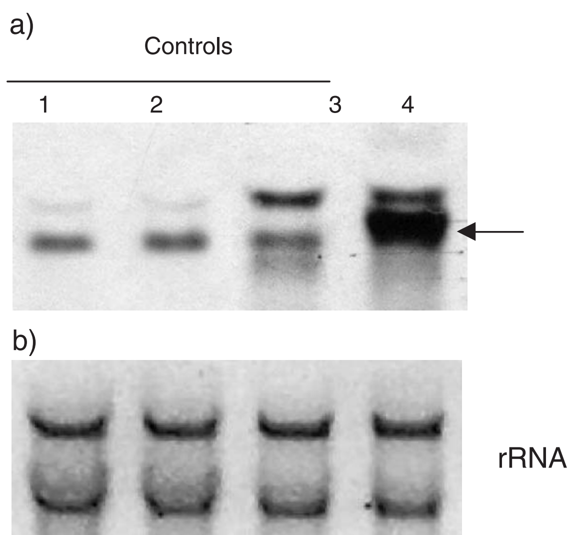

Fig. 2. Northern blot results. (a) mRNA from different cultures: lane 1—*K. marxianus* CCT 3172 with pKYES2 grown on 2% glucose; lane 2—*K. marxianus* CCT 3172 with pKY37 grown on 2% glucose; lane 3—*K. marxianus* CCT 3172 with pKYES2 grown on 2% galactose; lane 4—*K. marxianus* CCT 3172 with pKY37 grown on 2% galactose. (b) Sample control–rRNA before transfer to the nylon membrane.

expression vectors (Wésolowski-Louvel et al., 1996).

To construct a plasmid for the *GAP1* gene expression, the *S11* sequence was inserted as described above, in the pre-existing pY37 (Falcão-Moreira et al., 2000). pY37 was digested with *Sna*BI, the blunt end fragment *S11* was inserted into this vector and named pKY37. The insertion of the *S11* fragment into pYES2 and pY37 was confirmed by restriction map analysis and by sequencing with appropriate primers.

The estimation of plasmid stability revealed that, after 20 generations, 73 ± 2% of the cells contained the expression vector.

3.2. Characterization of K. marxianus transformed with the new vectors—growth and phenotype

Cell growth was estimated using glucose as carbon and energy source and the maximum specific growth rates are shown in Table 2.

Microscopic visualization of the cells harbouring p37 revealed both long and round shaped cells. When cells enter stationary phase in medium with galactose and in the presence of 200 mM of Ca^{2+}, it was observed that cells start to flocculate (Fig. 1a(i)). The wild type strain, in the same culture conditions, did not flocculate (Fig. 1a(ii)). Flocculation of the transformed cells overexpressing the *GAP1* gene was increased when the culture broth was renewed with galactose medium supplemented with 200 mM Ca^{2+} after sugar depletion (Fig. 1b).

3.3. Overexpression of the GAP1 gene

The *K. marxianus* cells transformed with pKYES2 and pKY37 were grown on YP + galactose and on YP + glucose media and harvested at O.D. 600 nm = 1. This cell density was chosen as it leads to an efficient cell disruption to extract the RNA (Ausubel et al., 1996). The expression of *GAP1* gene was evaluated by Northern analysis (Fig. 2). Two transcripts were detected in the control cells transformed with the empty vector grown either in glucose or galactose media. These transcripts correspond to the endogenous *GAP1* gene and to *GAP2/GAP3* (Fernandes et al., 1995). In the *K. marxianus* cells transformed with pKY37 grown in glucose, a similar result was observed. However, when galactose was used as carbon source, a third transcript was detected (marked with an arrow in lane 4), as a result of the induction of *GAP1* expression.

Cytosolic GAPDH activity was assessed in both cells. An increase of 20 ± 7% in the activity was determined for cells grown in YP + 2% galactose

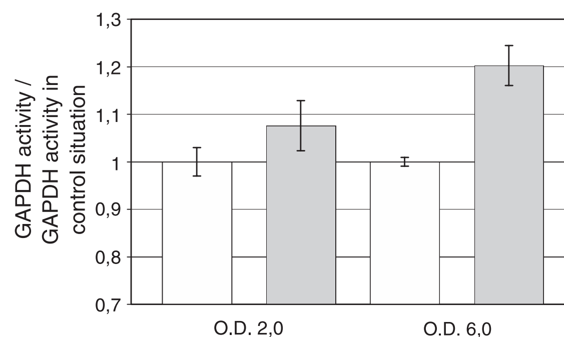

Fig. 3. GAPDH activity assays. The white columns refer to the control cells (transformed with the empty vector-control situation) and the gray columns refer to the transformed cells with pKY37. The cells were grown in YP galactose 2% media and harvested at a O.D. 600 nm of 2 and 6.

harvested at O.D. 600 nm = 6 (Fig. 3), confirming the induction of *GAP1* expression and subsequent translation into the functional GAPDH isoform in the cytosol.

3.4. Presence of p37 in the cell wall

By labelling the surface cell wall proteins with Sulfo-NHS-LC-Biotin followed by a streptavidin reaction, it was possible to detect differences in the cell wall protein composition between the wild type cells carrying the empty vector and the *GAP1* expressing vector (Fig. 4a). It is clear that p37 (indicated by the arrow) is present in the cell wall of both strains. However, on lane 2, a more intense protein band was visualised, which indicated an accumulation of p37 at the cell surface. p37 can be present in the cell wall in both glycosylated and non-glycosylated forms accounting for the detection of proteins with a molecular mass of 37 and 35 kDa, respectively (Fernandes et al., 1992).

A Western blot analysis was performed with the SDS-extractable cell wall proteins from cells grown in media containing 2% galactose, harvested at O.D. 600 nm = 4 (Fig. 4b). The p37 was immunodetected in both lanes 1 and 2, in agreement with the previous results shown in Fig. 4a. The amount of p37 (glycosylated + non-glycosylated forms) is increased in lane 2, supporting the evidence of an accumulation of p37 in the cell wall (as indicated by an arrow). As the antiserum used is a polyclonal, there is a cross reactivity and a protein with a higher molecular mass is also immunodetected.

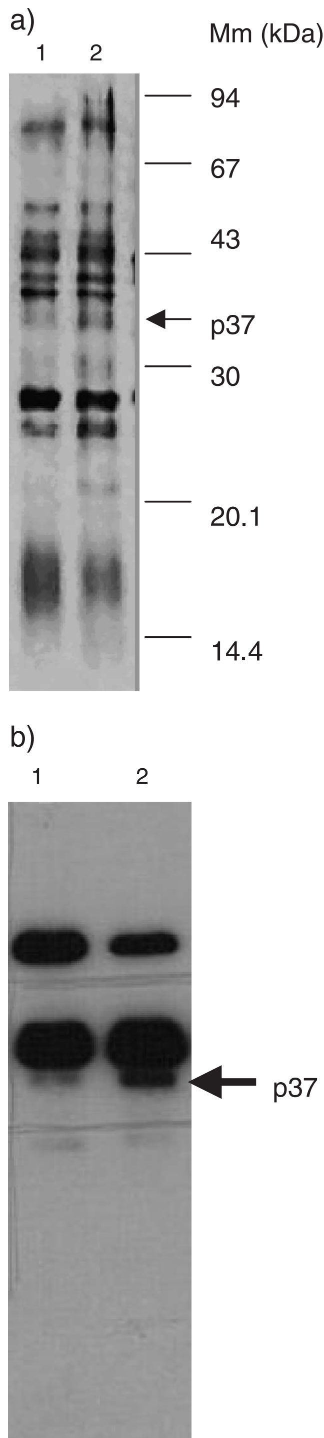

Fig. 4. (a) Cell wall surface biotinylated proteins from cells grown on YP + 2% galactose: lane 1—*K. marxianus* CCT 3172 with pKYES2; lane 2—*K. marxianus* CCT 3172 with pKY37. (b) (i) Immunodetection of p37. (a) Cell wall proteins from cells grown on YP + 2% galactose: lane 1—*K. marxianus* CCT 3172 with the pKYES2, lane 2—*K. marxianus* CCT 3172 with pKY37.

4. Discussion

A higher productivity is achieved in continuous bioreactors working at high cell density. The downstream processing of the secreted product would be more efficient if cells flocculate, thus avoiding the time-consuming steps of cell separation. Therefore, there is a growing interest in obtaining industrial

strains with a flocculent phenotype. To that end, we aimed at converting a non-flocculent strain of *K. marxianus* into a flocculent one by testing whether the accumulation of an isoform of glyceraldehyde-3-phosphate dehydrogenase (p37) in the cell wall could promote a flocculation phenotype. This strategy was based on previous results showing that the accumulation of p37 in the cell wall of non-flocculent *S. cerevisiae* led the cells to acquire a flocculation phenotype (Falcão-Moreira et al., 2000). A *GAP1* expression vector inducible by galactose and containing the *S11* origin of replication sequence was constructed and used to transform the *K. marxianus* non-flocculent strain CCT 3172. The results obtained from the transcript analysis indicated that the cloned *GAP1* gene is expressed in the presence of galactose as the energy and carbon source. The cytosolic GAPDH enzymatic activity increased 20% and a flocculation phenotype was observed. The relatively small size and number of the aggregates, when compared with the behaviour of the wild type flocculent strain *K. marxianus* ATCC 10022 (Sousa et al., 1992; Teixeira et al., 1989; Falcão-Moreira et al., 2000) can be related to the level of expression of *GAP1* induced by galactose.

The use of galactose to induce the gene expression in *Kluyveromyces* can impair flocculation in some strains. It has been reported that different sugars can have an inhibitory effect on flocculation, since they compete for the lectins in the cell wall surface (Stratford and Assinder, 1991). Géhin et al. (2001) found an inhibitory role for galactose in *K. bulgaricus* self-flocculation although galactose did not affect the flocculation phenotype of *S. cerevisiae* transformed with the pY37 (Falcão-Moreira et al., 2000). This fact supports that the flocculation phenotype acquired by the *Kluyveromyces* strain is related to the presence of p37 in the cell wall.

The molecular mechanisms underlying the ability of cells to aggregate when p37 accumulates in the cell wall is not fully elucidated. The presence of p37 in the cell wall can lead to changes at the surface, which is consistent to the alteration in calcofluor white sensitivity display by the mutant (Falcão-Moreira et al., 1998). The aggregation of the yeast cells may be facilitated by the exposure of p37 protein domains at the cell surface that easily interact with p37 in the cell wall of neighbouring cell.

In summary, we developed a *Kluyveromyces* expression vector harbouring the *GAP1* gene encoding the isoform of glyceraldehyde-3-phosphate dehydrogenase targeted to the cell wall leading to a flocculation phenotype. The results indicate that this is a useful tool to promote a flocculation phenotype. This leads to the possibility of developing high cell density flocculation bioreactors for protein production from yeasts.

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