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Construction of a flocculent brewer's yeast strain secreting *Aspergillus niger* β-galactosidase

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Abstract One way of improving heterologous protein production is to use high cell density systems, one of the most attractive being the flocculating yeast production system. Also, lactose is available in large amounts as a waste product from cheese production processes. The construction of flocculent and non-flocculent brewer's yeast strains secreting β-galactosidase and growing on lactose is presented. A plasmid was constructed coding for an extracellular β-galactosidase of *Aspergillus niger* and having, as selective marker, the yeast *CUP1* gene conferring resistance to copper. This selective marker allows for the transformation of wild-type yeasts. This work represents an important step towards the study of heterologous protein secretion by flocculent cells.

Introduction

The yeast *Saccharomyces cerevisiae* is recognised as a host for the production of heterologous proteins, especially those of eukaryotic origin. Furthermore, it is an organism generally regarded as safe; and yeast cultures are easy to handle and to scale-up. Only a few proteins are naturally secreted by yeasts, and this particular feature simplifies purification of heterologous products. However, the yeast expression system has a major drawback in the rather modest intrinsic secretory capacity of *S. cerevisiae*. It is possible to overcome this problem in several ways, such as by isolating supersecretory mutants (Sleep et al. 1991) or by optimising process parameters. One way to improve the economy of foreign protein production is the use of flocculent yeast cells. The use of flocculent yeast cells allows the cultivation to be carried out in continuous mode since, due to their sedimentation characteristics, flocculent cells can be retained inside the bioreactor. This allows for increased biomass concentration inside the bioreactor with a consequent increase in the concentration of product in the effluent. Moreover, the utilisation of flocculating cells gives an important contribution to the improvement of the separation process. Besides being less aggressive than other separation techniques, a reduction in the production cost is obtained since the amount of cells to be separated by centrifugation or filtration is reduced (Teixeira and Mota 1992). Furthermore, it is worth nothing that most recombinant products are currently produced by batch processes, even though continuous processes are usually more economical and efficient. Two major reasons are primarily responsible for this: one is the problem of plasmid loss, and the other is a contamination problem. The use of flocculent systems greatly reduces the risk of contamination and a proper design of the bioreactor with flocculent cells can overcome the problem of plasmid loss as has been shown for recombinant bacterial cells (Henry et al. 1990). It must be clearly pointed out that the use of high cell density systems for protein production has a clear advantage over other systems only if proteins are secreted into the medium. In this way, both system productivity and protein separation are highly enhanced.

It is our aim to study heterologous protein production/secretion by flocculent yeast cells. In this work, a plasmid harbouring both the *lacA* gene of *Aspergillus niger* coding for extracellular β-galactosidase and the *CUP1* gene coding for copper resistance was constructed. In addition, to allow studies concerning protein production, this plasmid can be used to transform commercial yeast strains either for the production of β-galactosidase, or for the conversion of waste from cheese production (lactose-containing whey) into ethanol.

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Strains constructed in this work will allow the performance of systems with flocculent and non-flocculent cells to be compared.

Materials and methods

Strains and media

The bacterial strain used for plasmid construction and DNA preparation was *Escherichia coli* DH5α which was grown according to Lennox (1955) in LB (1% casein, 0.5% yeast extract, 0.5% NaCl), containing 100 μg ampicillin (Ap)/ml. LB plates were supplemented with 2% agar. The *S. cerevisiae* strains were W204, a non-flocculent bottom fermenting brewer's yeast from Sapporo (obtained from Hefe Bank, Weihenstephan) and a transformant of W204 with the *FLO1* gene integrated into the genome (Watari et al. 1994), named W204-*FLO1*L (INT). Yeasts were grown in YEPG (1% Difco yeast extract, 1% Difco bactopeptone, 2% glucose). Selection and assays for copper resistance were carried out in NEP medium (Henderson et al. 1985) composed as follows: 0.2% $MgSO_4 \cdot 7H_2O$, 0.2% $(NH_4)_2SO_4$, 0.3% KH_2PO_4, 0.025% $CaCl_2 \cdot 2H_2O$, 0.2% Difco yeast extract, 0.3% Difco bactopeptone, 4% glucose or lactose and an appropriate volume of 20 mM $CuSO_4$ solution added to the cooled medium after sterilisation, to give the desired $CuSO_4$ concentration. SS medium (Teixeira et al. 1990) is composed as follows: 0.5% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.04% $MgSO_4 \cdot 7H_2O$, 0.1% Difco yeast extract, 2% glucose or other carbon source. The yeast plates were supplemented with 2% agar.

General DNA methods

Standard recombinant DNA methods were used (Maniatis et al. 1982). Enzymes were purchased from different manufacturers and were used as recommended. DNA fragments were isolated from agarose gels by using a QIAgen gel extraction kit. A method adapted from Holmes and Quigley (1981) was used for small-scale isolation of plasmid DNA from *E. coli*, followed by treatment with RNase A (75 μg/ml, 30 min, 37 °C). For large-scale extraction, QIAgen prep kits were used. *E. coli* was transformed by electroporation according to protocols from Bio-Rad.

Plasmid constructions

The plasmid pLD1 was constructed based on the plasmids pET13.1 (Henderson et al. 1985) and pVK1.1 (Kumar et al. 1992). Plasmid pET13.1 was digested with *Hin*dIII, treated with T4 DNA polymerase and alkaline phosphatase. Plasmid pVK1.1 was digested with *Sph*I, the 4.1-Kb fragment (*lacA* gene together with the *ADH1* promotor and terminator) isolated from 0.8% agarose gel electrophoresis and blunt-ended with T4 DNA polymerase. Plasmid pLD1 was obtained by joining cut pET13.1 with the 4.1-Kb fragment. The ligation mixture was used to transform *E. coli* to Ap resistance. The hybrid plasmid were isolated from Ap-resistant clones and identified by restriction analysis. It is worth noting that the original plasmid pET13.1 has two adjacent *Hin*dIII sites. In this construction only one of the sites has become blunt-end and hence the new vector pLD1 still has a *Hin*dIII. This has been verified by restriction analysis with *Hin*dIII.

Yeast transformation

Yeast was transformed according to the method of Ito et al. (1983) with modifications cited in Schiestl and Gietz (1989). The selective medium utilised was NEP-glucose-$CuSO_4$. The use of this selective medium enabled the selection of copper-positive clones and the indirect selection of positive Lac^+ transformants. Once the copper-positive clones were selected, they were maintained on NEP-lactose-$CuSO_4$ medium, i.e., imposing a double selection pressure for the plasmid pLD1.

β-Galactosidase activity assays

Screening for β-galactosidase activity in yeast clones

The presence of β-galactosidase activity in the yeast clones was tested by a microtitre plate assay with *p*-nitrophenyl-β-D-galactopyranoside (pNPG, Sigma) as substrate as previously described (Domingues et al. 1997). 100 μl of each cell culture were plated in a well and to each well was added 200 μl SDE (0.05 M Tris/HCl pH 7.5–8.0,0.01 M EDTA, 1 M KCl and 0.05 M 2-mercaptoethanol), a drop of 0.1% (w/v) SDS and a drop of chloroform. The β-galactosidase activity was detected by adding 50 μl of 4 mg pNPG/ml per well, incubating the plates at 65 °C for 30 min and detecting positive wells by their yellow colour.

Enzyme activity measurements

β-Galactosidase activity in the culture medium was assayed after concentrating the supernatant by ultrafiltration using Centrican-50 membrane (Amicon). The β-galactosidase activity was measured as the release of *p*-nitrophenol from pNPG. Samples were incubated with 1.7 mM substrate in 0.075 M Na-acetate buffer, pH 4.5, for 10 min at 65 °C. The pH was raised to 10 with 1 M Na_2CO_3 and the activity was measured spectrophotometrically at 405 nm on a scanning multiwell spectrophotometer (SLT Spectra; Bailey and Linko 1990). One unit of activity was defined as the amount of enzyme the hydrolysed 1 nmol pNPG/min at 65 °C.

Flocculation assay

Yeast cells were grown until the stationary phase. The cells were washed twice with 1.5% NaCl solution, pH 3.0. A 1 ml portion of the cell suspension was placed in a cuvette and the optical density (OD) was read at 620 nm. Then 40 μL of 100 mM $CaCl_2$, pH 3.0 was added and the solution was immediately mixed by inversion 18 times. Thereafter OD was measured at different time points (adapted from Soares et al. 1992). 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.

Shake flask cultures

Yeast cultures were carried out in 100 ml culture medium in 250-ml Erlenmeyer flasks at 30 °C with shaking (150 rpm). The flasks were inoculated with 1/10 volume of preinocula grown for 24–48 h at 30 °C. Although inocula were prepared as much as possible in a reproducible manner, small changes might have occurred.

Cell concentration

Cell concentrations were measured as dry-weight (DW) and/or OD. The DW was determined by filtering the sample through a 0.2-μm membrane which was then dried at 105 °C for 24 h. The OD was measured at 570 nm on a scanning multiwell spectrophotometer (SLT Spectra) and compared to a standard curve for OD versus biomass concentration previously constructed. For the flocculent strain, samples were treated with deflocculation solution (NaCl 1.5%, pH 3.0) before reading the OD.

Lactose, glucose, galactose and ethanol measurements

Total reducing sugar concentration was determined by the dinitrosalicylic acid method (Miller 1959). Lactose, glucose, galactose and ethanol concentrations were determined by HPLC (PL Hi-Plex Pb Column). The solvent used was ultrapure water, at a flow rate of 0.6 ml/min, while detection was effected with a refractive index detector. Temperature was maintained at 80 °C.

Results

Plasmid construction and yeast transformation

In order to express the *A. niger* β-galactosidase in yeast, the β-galactosidase encoding gene *lacA* was coupled to the *ADH1* promotor on the multicopy plasmid carrying also the selectable marker gene copper resistance, *CUP1*. The construction of plasmid pLD1 in described in Fig. 1.

The plasmid pLD1 was transformed to yeast using selection for copper resistance. Two brewer's yeast strains, W204, and the highly flocculent derivative strain, W204-*FLO1*L(INT), were selected as hosts and tested for copper resistance. Both strains grew at $CuSO_4$ concentrations of 1.9 mM in rich YEPG medium but both strains were sensitive to copper concentrations above 0.3 mM in NEP medium. A copper concentration of 0.35 mM was chosen for the selection of copper resistant transformants in NEP medium. The transformation efficiency for both W204 and W204-*FLO1*L(INT) was similar and varied over 15–35 transformants/μg DNA.

One of the main potential applications of these yeast transformants is their use in whey fermentation. For this reason, the copper resistance of the strains in the presence of the sugars galactose and lactose was tested in SS medium. Both strains grew at $CuSO_4$ concentrations of 0.2 mM but not at 0.5 mM. Consequently, the SS medium with lactose or galactose can be used as a selective medium for the transformants.

Flocculation properties of the strains

In order to analyse whether the flocculence of the recombinant strains was retained, a flocculation assay was performed. Figure 2 shows the sedimentation profiles of the flocculent host strain in NEP-glucose medium, and the recombinant strain in NEP-glucose-$CuSO_4$ and NEP-lactose-$CuSO_4$ media.

There is no difference in the sedimentation characteristics between the host strain W204-*FLO1*L(INT) and the recombinant strain W204-*FLO1*L(INT)/pLD1 in NEP-glucose medium. Nevertheless, there is a slight reduction in the flocculation ability of the recombinant strain in lactose medium. This could be the result of several factors that affect flocculation, since it is known that the ability of cells to flocculate is dependent on factors such as cell density, pH, sugar concentration,

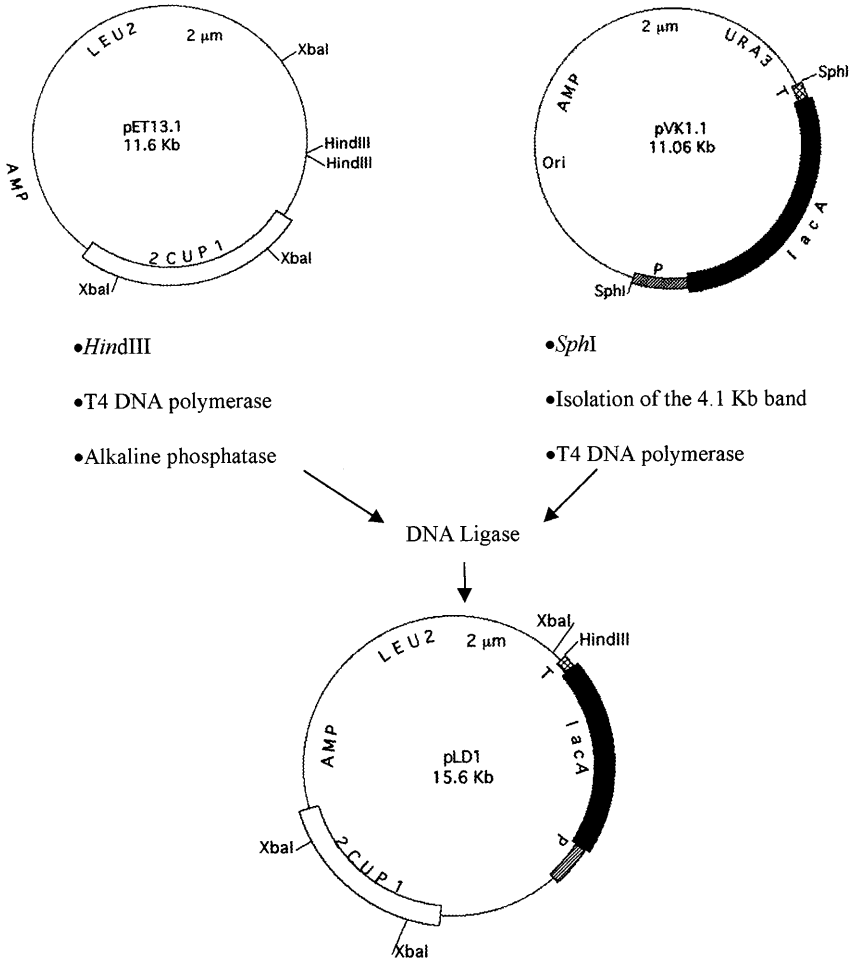

Fig. 1 Construction of the recombinant plasmid pLD1 containing the selectable marker *CUP1* and the *Aspergillus niger* β-galactosidase encoding gene *lacA* expressed from the yeast *ADH1* promotor. The *black box* indicates the *lacA* gene insert, the *hatched box* the *ADH1* promotor (*P*), the *crosshatched box* the *ADH1* terminator (*T*) and the *open box* indicates the *CUP1* gene

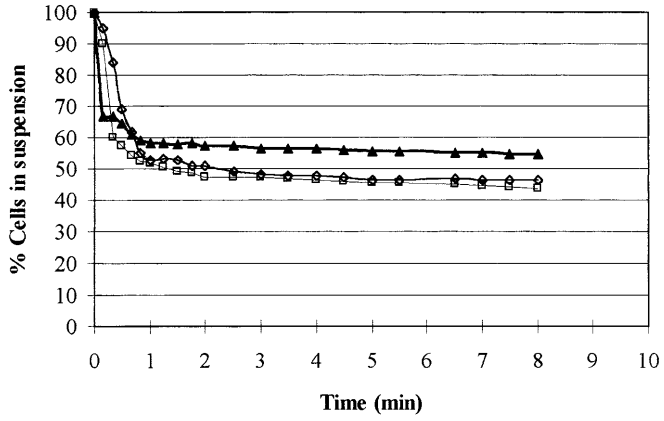

Fig. 2 Sedimentation profiles of W204-*FLO1*L(INT) in NEP-glucose medium (◇) and of the recombinant W204-*FLO1*L(INT)/pLDI strain in NEP-glucose-$CuSO_4$ (□) and NEP-lactose-$CuSO_4$ medium (▲)

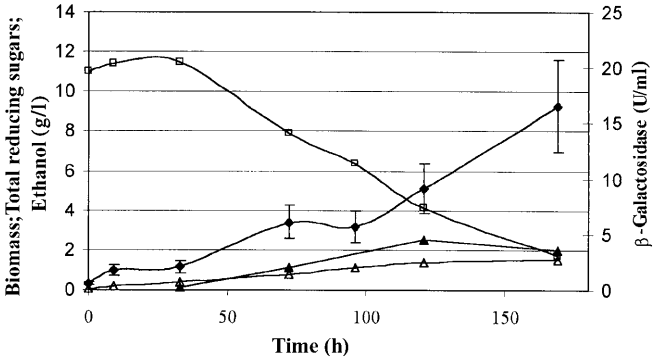

Fig. 3 Typical time course of batch culture in shake flasks using SS lactose (1%) medium for the recombinant strain W204-*FLO1*L(INT)/pLD1. Total reducing sugars (□), biomass concentration (△), ethanol concentration (△) and *β*-galactosidase activity (◆)

divalent ion concentration (namely Ca^{2+}) and monovalent ion concentration (Na^+ and K^+). Moreover, flocculation or brewer's yeast is inhibited specifically by sugars, namely mannose and mannose derivatives (Miki et al. 1982; Nishihara and Toraya 1987; Kihn et al. 1988). Previous evidence obtained with *Kluyveromyces marxianus* (Teixeira et al. 1995) indicates that lactose would not affect flocculation ability and the slight difference in flocculence behaviour in our experiments seems not to be due the presence of the plasmid pLD1. The recombinant strain has a similar sedimentation profile to that of the host strain in glucose medium.

Secretion of *β*-galactosidase

β-Galactosidase activity was detected in the culture supernatant of the yeast transformants but not in the supernatant of the host strains. A typical time course for a batch culture on SS lactose medium is presented in Fig. 3. *β*-Galactosidase activity in the culture medium increased with increasing cell concentration, reaching its maximum when cell growth approached the stationary phase.

In all shake flask cultures using lactose as the sole carbon source, lactose, glucose and galactose concentrations were monitored throughout the experiments. In the experiments with 1% lactose, glucose and galactose concentrations were found to be always below 0.8 g/l. The total reducing sugar profile presented in Fig. 3 thus closely represents lactose concentration in the medium. This suggests that once lactose is hydrolysed by *β*-galactosidase, glucose and galactose enter the cell with no significant accumulation in the culture broth and lactose hydrolysis is consequently the limiting step. However, when using 2% lactose concentration (as, for example in the experiment shown in Fig. 4), it was observed that the yeast culture was not able to metabolise all sugar in 200 h. The lactose disappearing rate is slow with no accumulation of glucose and galactose sugars in the first 120 h of the culture, indicating once again that the limiting step is the secretion rate of *β*-galactosidase up to this stage of fermentation. At a later stage of the culture, increasing amounts of glucose and galactose were detected in the culture medium. This

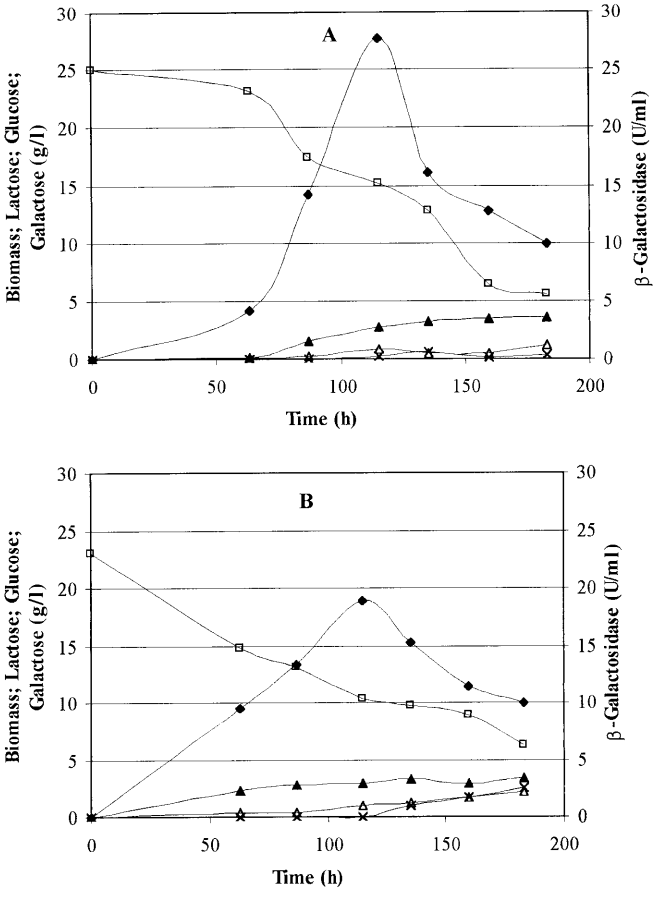

Fig. 4A, B Typical time course of batch culture in shake flasks using SS lactose (2%) medium for the recombinant strains W204/pLD1 (**A**) and W204-*FLO1*(INT)/pLD1 (**B**). Biomass concentration (▲), lactose concentration (□), glucose concentration (×), galactose concentration (△) and *β*-galactosidase activity (◆)

increase is most likely associated with lactose hydrolysis by β-galactosidase present in the supernatant, as sugars are not metabolised, as suggest by the absence of biomass growth. This may be caused by the ageing of the culture or by the absence of any nutrients other than the carbon source.

When comparing the two transformants strains, the non-flocculent W204/pLD1 and the flocculent W204/*FLO1*L(INT)/pLD1, there is no great difference in terms of β-galactosidase detected in the supernatant and in the lactose, glucose and galactose profiles as shown in Figs. 4 and 5. However, in terms of specific productivity (β-galactosidase/cell mass) the non-flocculent strain had repeatedly higher values than the flocculent strain (Table 1). This suggests that some β-galactosidase is retained inside the focus between the cell matrix after centrifugation and is not released to the supernatant. To confirm this hypothesis, after initial separation of cell biomass and supernatant, the biomass pellet was washed with Na-acetate buffer and recentrifuged: and the resulting supernatant was analysed for β-galactosidase. For the non-flocculent strain a residual β-galactosidase activity of less than 1% of the activity measured in the initial supernatant was detected after washing the pellet. For the flocculent strain values of 5–25% were obtained, with more residual β-galactosidase activity being obtained in the early stages of the culture. The residual β-galactosidase present in the washes of the non-flocculent strain is most probably protein bound to the cell wall while, in the case of the flocculent strain, β-galactosidase could be retained inside the cell matrix.

Secretion of β-galactosidase by the yeast transformants W204/pLD1 and W204-*FLO1*L(INT)/pLD1 using lactose or glucose as the sole carbon source was also compared. Both media had $CuSO_4$ at 0.35 mM as selective pressure. The secretion of β-galactosidase in lactose medium was found to be higher than in glucose medium, 17 U/ml and 8 U/ml respectively. This difference could be caused by selective pressure favouring a higher copy number of the plasmid on lactose medium.

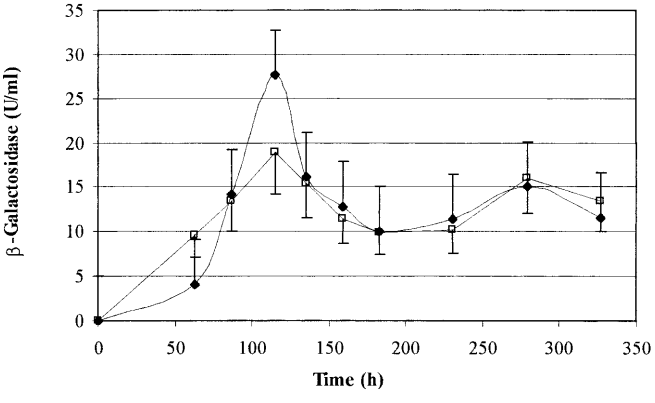

Fig. 5 Time course of β-galactosidase secretion by the two recombinant strains W204-*FLO1*(INT)/pLD1 (□) and W204/pLD1 (◆) in SS lactose medium (2%)

Table 1 Comparison of β-galactosidase secretion by the flocculent (W204-*FLO1*L(INT)/pLD1) and non-flocculent (W204/pLD1) transformats in terms of specific cellular product yield ($Y_{P/X}$; values at 115 h from Fig. 4)

Strain	$Y_{P/X}(U/g_{DW})$
W204-*FLO1*L(INT)/pLD1	0.79×10^5
W204/pLD1	1.07×10^5

Growth on selective medium

To study the effectiveness of lactose utilisation for growth by the recombinant strains, they were grown in shake flask cultures in selective medium containing either lactose or glucose as the carbon source. Both transformants, the flocculent W204-*FLO1*L/pLD1 and the non-flocculent W204/pLD1, showed a decrease in their specific growth rate ($\mu \sim 0.16$/h in lactose selective medium and $\mu \sim 0.18$/h in glucose selective medium) when compared to the respective host strains, W204 and W204-*FLO1*L(INT) ($\mu \sim 0.29$/h in glucose medium). It is well known that the synthesis of foreign gene products can be burdensome or toxic to the host cell, therefore poor plasmid stability and a low growth rate of recombinant cells are usually found (Yang and Shu 1996).

Although slow, the growth of the transformants on selective medium is comparable to growth rates of other relevant recombinant *Saccharomyces* strains on lactose-selective medium (Table 2).

Discussion

In this study the construction of a plasmid harbouring both the *lacA* gene encoding *A. niger* β-galactosidase and the *CUP1* gene encoding copper resistance is reported. This plasmid can be employed for the transformation of wild-type yeasts for commercial use, either for the production of β-galactosidase, or for lactose or whey consumption and the production of ethanol. The results presented clearly show that the recombinant brewer's yeast secretes active β-galactosidase into the culture medium and grows on lactose medium. It is also shown there are no differences in the flocculation ability between host and recombinant strain. These results contradict results previously reported (Domingues et al. 1999), where a reduction in flocculation ability of the recombinant strain was observed. In that case, the cloning of a lactose permease may justify the observed reduction in flocculation, since this protein is a cell membrane protein. As flocculation is a cell wall phenomenon, introducing a new protein into the cell membrane may interfere with cell-to-cell interaction. Previous results have described partial secretion by a laboratory strain of *S. cerevisiae* of this β-galactosidase expressed from a multicopy plasmid and the *ADH1* promotor (Kumar et al. 1992). We now show that by using the dominant selection marker *CUP1*, industrial strains can also be

Table 2 Comparison of recombinant lactose utilising *Saccharomyces cerevisiae* strains on lactose selective media. *Td* Doubling time

Cloned genes	Gene origin	Td (h)	Reference
LAC4, *LAC12*, *LAC13*	*Kluyveromyces lactis*	Very slow	Jeong and Vieth (1991)
LAC4, *LAC12*	*K. lactis*	7	Sreekrishna and Dickson (1985)
LAC4, *LAC12*	*K. lactis*	2.2	Rubio-Teixeira et al. (1998)
LAC4, *LAC12*	*K. lactis*	5 (non-adapted) 2–3 (adapted)	Domingues et al. (1999)
lacA	*Aspergillus niger*	8	Kumar et al. (1992)
lacA	*A. niger*	5	Present work

transformed and secrete β-galactosidase. This allows growth on lactose as the sole carbon source. At higher lactose concentrations, glucose and galactose accumulated. It is envisaged that their consumption can be improved by providing the yeast with more nutrient.

Besides this application, the main achievement using this plasmid is the possibility to study the secretion of heterologous proteins by flocculent yeasts in bioreactor cultivation. The recombinant strains constructed in this work will allow comparison of protein production by flocculent and a non-flocculent strain having otherwise the same genetic background. The results obtained suggest there are no significant differences in β-galactosidase secretion levels between the two strains. The β-galactosidase levels obtained with the highly flocculent brewer's yeast constructed in this work appear to be similar to those previously reported (Kumar et al. 1992; Ramakrishnan and Hartley 1993). The slight difference found in the specific cellular β-galactosidase activity between the non-flocculent and flocculent strains is most likely due to a retention of some of the protein inside the floc matrix. This suggests that at the phase of highest production, β-galactosidase accumulation occurs inside the floc and that the protein is able to diffuse through the porous floc afterwards. Using polymers that increase floc porosity (Lima et al. 1992) the β-galactosidase accumulation could be minimised. These results are encouraging, in terms of the secretion of biological macromolecules by flocculent cells since, for the large β-galactosidase, no significant differences were found between the flocculent and non-flocculent cells. This raises new perspectives for the production of heterologous proteins by *S. cerevisiae*. The limitations of low secretion levels are overcome by using continuous high cell density bioreactors with flocculating cell cultures. When considering the advantages of continuous high cell density systems for protein production, the secretion of the protein of interest represents a major aspect, as not only is the global volumetric productivity improved but also the protein separation processes are greatly simplified. For protein production, the cloning of an extracellular β-galactosidase would be a more attractive approach than the cloning of intracellular β-galactosidase as published in a previous work (Domingues et al. 1999). However, as concerns lactose fermentation, the intracellular β-galactosidase cloning might be a better approach because the lactose is hydrolysed as it enters the cell. For extracellular β-galactosidase, the lactose hydrolysis occurs outside the cell and might lead sugar inhibition problems, greatly depending on the secretion/hydrolysis rate.

This is the first time to our knowledge that a flocculent yeast strain has been constructed for foreign protein secretion.

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