Development of Stable Flocculent *Saccharomyces cerevisiae* Strain for Continuous *Aspergillus niger* β-Galactosidase Production

Carla Oliveira,¹ José A. Teixeira,¹ Nelson Lima,¹ Nancy A. Da Silva,² and Lucélia Domingues¹*

IBB-Institute for Biotechnology and Bioengineering, Centre for Biological Engineering, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal¹ and Department of Chemical Engineering and Materials Science, University of California, Irvine, CA 92697-2575, USA²

Received 15 November 2006/Accepted 9 January 2007

A flocculent *Saccharomyces cerevisiae* strain was engineered to stably secrete *Aspergillus niger* β-galactosidase in a continuous high-cell-density bioreactor. The δ-sequences from the yeast retrotransposon Ty1 were used as target sites for the integration of the β-galactosidase expression cassette. High-copy-number transformants were successfully obtained using the δ-integration system together with the dominant selection antibiotic, G418. The integration of multiple copies was confirmed by genomic Southern blot analysis. Integrants with the highest β-galactosidase levels (approximately eight gene copies) had similar β-galactosidase activities as a recombinant strain carrying the β-galactosidase expression cassette in a YEp-based vector. The β-galactosidase expression cassettes integrated into the yeast genome were stably maintained after eight sequential batch cultures in a nonselective medium. In continuous high-cell-density culture under the same operating conditions, the integrant strain was more stable than the plasmid-carrying strain. To our knowledge, this is the first study of multicopy δ-integrant stability in a continuous bioreactor operating at different dilution rates.

[Key words: genetic stability of delta-integrating systems, continuous high-cell-density culture, *Aspergillus niger* β-galactosidase production, recombinant *Saccharomyces cerevisiae*, yeast flocculation]

β-Galactosidase (β-D-galactoside galactohydrolases; EC 3.2.1.23) can cleave β-linked galactose residues from various compounds and is commonly used to cleave lactose into galactose and glucose. This enzyme is thus widely used in the food and pharmaceutical industries. The economic interest in β-galactosidase is closely related to some unwanted lactose properties, namely, its poor digestibility, low sweetness, power, low solubility, and low biodegradability.

The potential of flocculent *Saccharomyces cerevisiae* to produce and secrete active *Aspergillus niger* β-galactosidase from multicopy expression plasmids was demonstrated previously (1). Although the utilized expression system had a good plasmid stability in batch and fed-batch culture systems (2), plasmid loss in a nonselective medium led to a decrease in productivity during continuous culture operation particularly at high dilution rates (3). The interest in the recombinant strains that have been developed to date for *A. niger* β-galactosidase production lies not only on the high expression levels (1) but also on the flocculation ability of the recombinant strains. The ability of these recombinant strains to aggregate and form flocs combined with an appropriate bioreactor design that retains the yeast flocs inside the bioreactor allows for the operation of a continuous high-cell-density system leading to increased volumetric productivity (4). Moreover, the utilization of flocculating cells provides an important contribution to the improvement of downstream processing (5, 6). However, to take proper advantage of these systems, strain stability is needed so that continuous operation does not lead to a progressive loss of the product. Such stability is normally achieved by integrating a recombinant DNA into the genome. When utilizing the classical integrative YEp-type vectors, a single-copy gene (e.g., *URA3, TRP1*) is often used as the homologous integration site resulting in low-copy-number integration, generally one or two per cell (7).

To increase the number of the integrated copies of the desired gene, one may use repetitive sequences in the yeast genome as target sites for homologous recombination. Research studies on the multiple copy integration for heterologous protein production have been mainly carried out for rDNA (8, 9) and δ-sequences (10–13). The δ-sequences flank the regions of the Ty1 retrotransposon of *S. cerevisiae*. There are approximately 342 δ-sequences, including both solo and flanking Ty1 elements, in the haploid yeast genome (14, 15) allowing multiple gene copy integration, particularly when a dominant selection marker is used. Several recombinant proteins have been successfully expressed using the δ-integrative systems. In most of the reports, the integration of the target gene occurred in one or two tandem arrays. As a consequence, stability should be carefully monitored in long-term cultivation as gene copies may be looped-
out through excisional recombination. According to the literature, the stability of the tandem integrations seems to be highly dependent on the target gene.

In this study, we aim to construct stable flocculent *S. cerevisiae* strains producing and secreting *A. niger* β-galactosidase that can be used in a continuous bioreactor. The *A. niger lacA* gene was expressed under the control of the ADH1 promoter and terminator, integrated into the *S. cerevisiae* genome using a α-integrative vector, and compared with the expression system based on a YEp vector previously used (1). One of the constructed strains was tested in a continuous high-cell-density production system and its stability was evaluated at different dilution rates.

**MATERIALS AND METHODS**

**Strains and plasmids** Escherichia coli DH5α (F− recA1 endA1 thi-1 gyrA96 relA1 supE44 thi-1 Φ80lac<sup>ΔM15</sup> lacI<sup>∆Z</sup>) was used for plasmid maintenance and construction. *S. cerevisiae* strain NCYC869-<wbr/>wt (Mata <wbr/>Flo) was used as a recipient strain for transformation. The construction of the recombinant *S. cerevisiae* NCYC869-A3/pVK1.1 flocculent strain was previously described (1). The lacA expression cassette containing the lacA gene (coding for *A. niger* β-galactosidase) flanked by the ADH1 promoter and terminator was isolated from plasmid pVK1.1 (16). This expression cassette includes the native secretion signal for *A. niger* β-galactosidase.

Plasmid pO-neo (13) was used as carrier vector for the integration of the lacA expression cassette into the yeast chromosomes. This is a δ-integrating vector containing the bacterial neu<sup>+</sup> gene for selection against G418, an aminoglycoside antibiotic.

**Media and cultivation** LB medium (0.5% NaCl, 0.5% yeast extract, and 2% tryptone), pH 7.5, supplemented with 100 µg of ampicillin/ml was used for *E. coli* cultivation. YPD medium (2% peptone, 1% yeast extract, and 2% glucose) supplemented with increased concentrations of filter-sterilized G418 (ranging from 0.2 to 1.5 g/l) was used for the selection and maintenance of the yeast transformants harbouring the G418 resistance gene. Culture medium plates were supplemented with 2% agar. For the selection of transformants after electroporation, selective plates were supplemented with 1 M sorbitol. To select for β-galactosidase producing colonies, YPD medium was supplemented with 40 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). LB and YPD plates were incubated at 37 and 30°C, respectively.

Standard β-galactosidase activity assays were conducted in 13 × 160-mm culture tubes containing 5 ml of YPD medium inoculated with a loop full of cells and the β-galactosidase activity was measured after 24 h of incubation.

Batch cultures for growth rate determination were carried out in 500-ml flasks containing 100 ml of YPD medium (1% glucose).

Sequential batch cultures for stability studies were conducted in 250-ml flasks containing 20 ml of YPD medium. In each batch, cells were cultivated to the late exponential phase (approximately 10 generations) and a 1% (v/v) inoculum was used between cultures. After 80 generations, the cells were deflocculated with 15 g/l NaCl, pH 3.0, spread on YPD plates, and incubated at 30°C. Some of the resulting colonies were transferred to the appropriate selective medium plates. All tubes and flasks were incubated at 30°C with shaking at 150 rpm.

**DNA manipulations** All restriction enzymes, Klenow fragment, and alkaline phosphatase were purchased from Fermentas International (Burlington, Ontario, Canada). T4 DNA ligase was purchased from Promega Corporation (Madison, WI, USA), and BglII (12 bp) and SphI (8 bp) linkers were obtained from New England Biolabs (Beverly, MA, USA).

DNA fragments were purified from agarose gels using the Qiagen Gel Extraction kit (Qiagen, Hilden, Germany). Large-scale plasmid DNA preparation from *E. coli* was carried out using the Genopure Plasmid Maxi kit (Roche Diagnostics, Basel, Switzerland).

**Plasmid construction** To integrate the β-galactosidase expression cassette into the genome of *S. cerevisiae*, a vector based on the pO-neo vector (13) was constructed (Fig. 1). The lacA expression cassette (4.1 Kb) was isolated from plasmid pVK1.1 (16) by SphI restriction digestion. Using a linker, the unique SacI site on pO-neo was changed to a unique SphI site and the lacA cassette was cloned into the modified restriction site. The unique AccI site within the delta sequence in the pO-neo vector was changed by BglII by a linker as the enzyme Acel cuts the lacA gene. The constructed vector was named pCOI and was linearized with BglII before yeast transformation.

**Yeast transformation** Yeast integrative transformation with the δ-integrating vector was performed by electroporation. The preparation of electrocompetent yeast cells was conducted as described previously (17). Yeast cells (50 µl) were gently mixed with 10 µg of linearized DNA and transferred into the electroporation cuvettes (0.2 cm electrode; Bio-Rad Laboratories, Hercules, CA, USA). An electric pulse of 1.5 kV and 200 µs was applied with a capacitance of 25 µF, using Gene Pulse X-Cell (Bio-Rad Laboratories). After the application of electric shock, 1 ml of YPD medium supplemented with 1 M sorbitol was added to the cell suspension and the mixture was incubated for 3 to 16 h at 30°C with shaking at 85 rpm. Following the incubation, the cells were deflocculated (Bio-Rad Laboratories) and plated on selective YPD-G418 plates.

**Analysis** The extracellular β-galactosidase activity was determined by measuring the release of p-nitrophenol from p-nitrophenyl-β-D-galactopyranoside (pNPG) as previously described (1). Samples were incubated with 1.7 mmol/l substrate in 0.075 mol/l Na-acetate buffer, pH 4.5, for 10 min at 65°C. The pH was increased to 10 with 1 mol/l NaCO<sub>3</sub>, and the absorbance was measured at 405 nm. One unit of activity was defined as the amount of enzyme that hydrolysed 1 nmol pNPG per min at 65°C. Total reducing sugar concentration was determined by the dinitrosalicylic acid method (18).

Biomass concentrations were measured as dry weight or using the absorbance method. The dry weight was determined by filtering the sample through a 0.2-µm filter paper and then drying at 105°C for 24 h. In the absorbance method, the cells were deflocculated and diluted with 15 g/l NaCl, pH 3.0, and the 600 nm absorbance values were converted to dry weight using a previously constructed standard curve.

Cell viability was estimated by the vital methylene blue staining method and direct cell counting using a Neubauer chamber. The percentage of cells expressing β-galactosidase was determined on the YPD plates containing X-gal as an indicator of β-galactosidase activity.

**Southern blot analysis** The integration copy number and patterns of the β-galactosidase expression cassette were analysed by Southern blot. Total genomic DNA was isolated according to the method of Hofman and Winston (19) with the modifications referred to in the method of Ausubel et al. (17), and was digested with XbaI restriction endonuclease, which cleaves the DNA once in the integrative vector 2255 bp upstream of the beginning of the lacA gene. After agarose (0.8%) gel electrophoresis, the separated DNA fragments were transferred onto a positively charged nylon membrane (Roche Diagnostics) and fixed to the membrane by a 30’s exposure to UV light using a UV Cross Linker (Bio-Rad Laboratories). Probes were labelled using the DIG-Labeling and Detection kit (Roche Diagnostics) and prehybridization, hybridization, and subsequent nonradioactive colorimetric detection were
carried out following the kit protocol. Hybridization was carried out at 47°C for 16 h.

To calculate the integration copy number for tandem arrays, two different methods were used. Both methods estimate the copy number of the tandemly integrated plasmid by measuring the relative ratio of intensities of the multicopy band to a single-copy band. In the method that we designated as the direct method, this comparison was made between the darker band exhibiting the plasmid size and the lighter band that contains the $lacA$ sequence at one end of the tandem copies (13, 20). For this method, a single $lacA$ probe was needed for the Southern blot analysis. The $lacA$ probe was a 633 base pair (bp) $EcoRI$ fragment containing part of the $lacA$ gene from plasmid pVK1.1. In the other method that we designated as the indirect method, the single-copy band corresponds to the hybridization of a reference gene that is known to be present as one copy in the genome (21–23). With this method, two different probes may be used. In this work, a 505 bp $URA3$ probe was simultaneously used with the 633 bp $lacA$ probe as both probes showed similar lengths and GC contents. The $URA3$ probe was a 505 bp $AclI$ fragment containing part of the $URA3$ gene from the YIp5 plasmid (Stratagene, La Jolla, CA, USA). The $URA3$ gene was used as a standard probe for one gene copy as there is only one copy of the $URA3$ gene in the haploid $S. cerevisiae$ genome. Quantity One Software (Bio-Rad Laboratories) was used to estimate the integrated copy number for the inserted gene by the two methods.

Continuous-culture experiments An airlift bioreactor of the concentric draft tube made of Perspex with a working volume of 6.5 l was used (3). The regulation system allows for: temperature control at 30±1°C; foam level control by the addition of antifoam (A-5551; Sigma-Aldrich Corporation, St. Louis, MO, USA); and pH control by the automatic addition of ammonia, with the set-point fixed at pH 4.0±0.1. The system was aerated with filtered air at a flow rate range of 0.0800 to 1.0000±0.0002 vvm (volume air per volume reactor per minute). Flow rate was controlled using a Hastings mass-flow controller. The dilution rates were between 0.1 and 0.33 h⁻¹. The semi-synthetic lactose medium (SSLactose), containing 0.5% $KH_2PO_4$, 0.2% $(NH_4)_2SO_4$, 0.04% $MgSO_4$·7$H_2O$,
0.2% yeast extract, and 5% lactose, was used as substrate.

The clone 7 integrant was selected for the continuous-culture experiments. For the start-up of the continuous culture, cells were grown overnight in a 250-ml shake flask containing 100 ml of SS lactose medium and this culture was used to inoculate a 2-1 Erlenmeyer flask filled with 1/2 culture medium. After 24 h, the cell suspension was aseptically transferred to the bioreactor, which was maintained in batch operation for 24 h before switching to continuous feeding.

RESULTS

Chromosomal integration of lacA gene  A linearized pCO1 plasmid was introduced into S. cerevisiae strain NCYC869 and transformants were selected on YPD plates with different G418 concentrations (ranging from 0.2 to 1.5 g/l), 1 M sorbitol, and X-gal for blue screening of the transformants expressing β-galactosidase. As previously observed (12, 13, 22, 24, 25), the number of transformants obtained was largely dependent on the G418 concentration added; this number decreased with an increase in the G418 concentration. Furthermore, a longer incubation after yeast transformation prior to G418 selection was extremely important for transformant recovery, increasing the final number of transformants obtained. The colonies with a deep blue colour, expressing higher β-galactosidase activity, were screened by a standard β-galactosidase activity assay and the colonies with higher expression levels were further studied (Table 1). As shown in Table 1, the selected integrants presented β-galactosidase activity similar to that obtained with the multicopy YEp-based vector (pVK1.1). Independent of the G418 concentration used, a range of enzyme production levels was obtained. Wang et al. (13) obtained similar results; although the average number of integrations per strain was greater on the plates with higher concentrations of G418, high-copy-number strains appeared at all G418 concentrations.

Four transformants from each G418 concentration (0.2, 0.5, 0.8, and 1 g/l) and six transformants selected from the 1.5 g/l G418 plates were analysed by Southern blot. The pattern for a single integration site with a tandem array of integrants was most frequently observed, but two-site integration patterns with various copy numbers were also detected. The transformants typically presented different sites of integration (data not shown). Consistent with the β-galactosidase standard activity assay, high-copy-number integrants were observed at all G418 concentrations used for the selection. However, note that the transformants were screened on the X-gal selective plates and the colonies with a deep blue colour were picked out, preselecting for high expression levels.

The difference in the values obtained by the two methods used in the determination of the copy number was within the 20% relative error attributed to copy number estimation in quantitative Southern blot analysis (13). Taking this into account, the mean value of both methods was used for the determination of the integration copy number of the selected transformants (Table 2). The clones with higher β-galactosidase activity had seven to eight gene copies integrated in the yeast genome.

<table>
<thead>
<tr>
<th>Strain/Clone number</th>
<th>β-Galactosidase activity (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prior to long-term cultivation</td>
<td>After eight sequential batch cultures</td>
</tr>
<tr>
<td>S. cerevisiae NCYC869-A3/pVK1.1</td>
<td>212±18</td>
</tr>
<tr>
<td>38 (0.2 g/l G418)</td>
<td>244±20</td>
</tr>
<tr>
<td>52 (0.5 g/l G418)</td>
<td>214±45</td>
</tr>
<tr>
<td>7 (1.5 g/l G418)</td>
<td>240±45</td>
</tr>
<tr>
<td>9 (1.5 g/l G418)</td>
<td>218±19</td>
</tr>
</tbody>
</table>

These clones were obtained after the transformation of S. cerevisiae NCYC869-wt with vector pCO1 and selected at different G418 concentrations as indicated.

<table>
<thead>
<tr>
<th>Clone number</th>
<th>Non-tandem copy number</th>
<th>Tandem copy number</th>
<th>Total copy number</th>
</tr>
</thead>
<tbody>
<tr>
<td>38 (0.2 g/l G418)</td>
<td>2</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>52 (0.5 g/l G418)</td>
<td>2</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>7 (1.5 g/l G418)</td>
<td>2</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>9 (1.5 g/l G418)</td>
<td>1</td>
<td>6</td>
<td>5</td>
</tr>
</tbody>
</table>

These clones were obtained after the transformation of S. cerevisiae NCYC869-wt with vector pCO1 and selected at different G418 concentrations as indicated.

Characterization of integrated gene expression

Copy number and β-galactosidase activity relationship

The relationship between the copy number of the lacA gene integrated into the S. cerevisiae genome and the extracellular β-galactosidase activity was studied. Integrants with one and two dispersed copies were obtained with another integrative vector (Ylp5+ lacA expression cassette). For the higher copy numbers (up to eight), integrants were obtained with the vector pCO1 and the copy number was estimated by quantitative Southern blot analysis. In Fig. 2, a linear relationship (R²=0.994) can be observed up to a copy number of eight.

Effect on specific growth rate

For the integrants presenting higher β-galactosidase activity (clones 7 and 38), growth rate studies were performed in YPD medium. The growth rates determined for the δ-integrants were within the 95% confidence interval of the growth rate determined for the host strain (0.18±0.06 h⁻¹). Therefore, the dispersed δ-mediated integrations (at least to a copy number of eight) had no significant effect on growth.

Stability evaluation of recombinant strains

The structural stability of the δ-integrations was analysed for two different clones with a higher copy number: clone 7 with eight copies and clone 52 with seven copies (Table 1). After eight sequential batch cultures in YPD medium (with approximately ten generations per culture), a sample was spread on YPD plates, and 50 colonies from each clone were randomly selected for analysis in the YPD selective medium containing G418 and X-gal. Each of the colonies tested was resistant to G418 and showed a blue color indi-
were started at 0.1 h after eight sequential batch cultures. Continuous cultures were carried out with clone 7 to confirm its stability under these operating conditions. Clone 7 was selected as it presented the highest copy number and copy number maintenance was confirmed (data not shown). Clone 7 was measured values for all clones were within the 95% confidence interval of the original value (Table 1). After the Southern blot analysis of three colonies of each clone tested, copy number maintenance was confirmed (data not shown).

**Continuous-culture experiments** Continuous high-cell-density cultures were carried out with clone 7 to confirm its stability under these operating conditions. Clone 7 was selected as it presented the highest copy number and β-galactosidase expression values as well as integration stability after eight sequential batch cultures. Continuous cultures were started at 0.1 h⁻¹ dilution rate and increased stepwise (up to 0.33 h⁻¹). For each tested dilution rate, five residence times were allowed to be completed. At the end of this process, fermentation parameters (i.e., cell viability, percentage of cells expressing β-galactosidase, total reducing sugar concentration, biomass, and β-galactosidase concentration) were measured and a new dilution rate was applied. During the operation, cell viability was more than 95% and the total reducing sugar concentration was below 2.5 g/l indicating total lactose consumption at all tested dilution rates. In all conditions, the δ-integrator presented higher specific β-galactosidase activity than the multicopy YEp-based vector-carrying strain. The main results in regard to strain stability are summarised in Table 3. During the continuous cultures, the percentage of cells expressing β-galactosidase was higher for the integrant strain than for the strain carrying the plasmid pVK1.1. For the plasmid-carrying strain, 60% white colonies were observed in the X-gal plates (at 0.35 h⁻¹ dilution rate); this number decreased to 5% with the integrant continuous culture. However, note that for the integrant strain, different blue patterns were observed in the X-gal Petri plates and all colonies were counted as β-galactosidase producing cells. The different blue patterns observed in the Petri plates may indicate population heterogeneity in regard to gene copy number. Nevertheless, this heterogeneity is also observed for the plasmid-based system as the plasmid copy number varies in the population.

**DISCUSSION**

A flocculent *S. cerevisiae* strain secreting high levels of *A. niger* β-galactosidase was previously constructed (1). However, when used in a continuous high-cell-density fermentation system, at high dilution rates, the recombinant strain became unstable leading to a heterogeneous population inside the bioreactor (3). To take full advantage of the yeast flocculation ability, the expression cassette coding for β-galactosidase expression and secretion should be integrated into the yeast genome.

We previously constructed a Ylp5-based vector for integrative β-galactosidase expression. However, the enzymatic activity was very low and low-copy-number integration was confirmed (data not shown). This result suggests that multiple copies of the lacΔ gene are necessary to produce *A. niger* β-galactosidase at sufficiently high levels similar to the strain carrying the YEp-based plasmid.

The use of the δ-integration system has been reported to enable increased production of heterologous proteins (12, 13) by increasing the number of gene integrations in the yeast genome. Therefore, this multiple δ-integration system was employed to introduce the lacΔ gene into the flocculent *S. cerevisiae* strain to overcome the limitations of the previously constructed strain mentioned above.

Integrants with copy numbers of up to eight were obtained. High-copy-number integrants were obtained regardless of the G418 concentration used in the selective medium. This corroborates previous results showing that high-copy-number strains appear at all G418 concentrations (13). Moreover, Parekh *et al.* (12) have reported the effect of chromosomal position on the expression of the bacterial neo gene. A low G418 resistance clone with high integrated neo copy number was obtained by these authors.

A linear correlation between β-galactosidase activity and integrated copy number has been obtained (Fig. 2). Because different integration sites have been observed, this linear correlation indicates that the integration on different δ-sites

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dilution rate (h⁻¹)</th>
<th>β-Galactosidase (U/g biomass)</th>
<th>Percentage of cells expressing β-galactosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. cerevisiae</em> NYCY869-A3/pVK1.1</td>
<td>0.13</td>
<td>5.1E4</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>0.24</td>
<td>4.8E4</td>
<td>Not determined</td>
</tr>
<tr>
<td></td>
<td>0.35</td>
<td>3.2E4</td>
<td>40</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> NYCY869-wt/pCO1–Clone 7</td>
<td>0.10</td>
<td>9.0E4</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>7.0E4</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>0.33</td>
<td>5.2E4</td>
<td>95</td>
</tr>
</tbody>
</table>
but not alter gene expression. This finding corroborates previously reported data showing that insertion into δ-sequences has no negative effect on expression (26).

The number of integrated gene copies reported in the literature using this system is relatively variable, ranging from 3 (10, 23) to 44 (22). Apparently, the copy number obtained is highly dependent on the size and content of the expression cassette (24). Note that for the expression of E. coli β-galactosidase, Lee and Da Silva (24) obtained nine integrated copies using the same vector (p6-neo) for construction. When the size of the integrating vector was reduced, the copy number of integrated lacZ expression cassettes could be further increased to 18.

Even though hundreds of δ-sequences are randomly distributed in the genome (14), multiple tandem integration occurred only at a few sites (1–3 sites) as confirmed by Southern blot analysis and also as reported by others (13, 20). Kim et al. (20) suggested that this finding indicates that the target sites of δ-mediated homologous recombination are selected on a particular region of a chromosome as hot spots. It seems that the first integration of a plasmid at a δ-sequence may accelerate the next integration event at the same site because more δ-sequences are available around the first target site, making the first integration region a hot spot.

For the integration of multiple gene copies, it is extremely important to check for stability as the integrated copy number may decrease by gene loop-out events. Transformant stability evaluation is typically carried out by the analysis of sequential batch cultures in a nonselective medium. In some works, only the G418 resistance is assessed (22, 25, 27). This is clearly insufficient to address the stability of transformants as a decrease in copy number may not affect the G418 resistance. Other studies address the stability of transformants by assessing copy number maintenance by Southern blot analysis (13, 20) or by measuring the activity of the cloned protein (23, 24, 28). In only one study, stability was assessed in a chemostat culture aside from a sequential batch culture (20). On the basis of xylitol production level, the authors concluded that the constructed strain is stable both in the chemostat culture for 10 d at 0.04 h⁻¹ dilution rate (more than 160 generations), and in the sequential batch culture (more than 336 generations).

Typically, for stability evaluation, Southern blot analysis is conducted after 70–100 generations in a nonselective medium. The stability of the constructed strains described in this work was evaluated after 80 generations, corresponding to eight sequential batch cultures, using the procedure described in the literature (24). G418 resistance, β-galactosidase activity, and copy number maintenance were evaluated making it possible to conclude that, in batch processes, the studied recombinant strains are stable. It was also confirmed that when operating in the continuous mode at high-cell-density, the selected strain is more stable than the strain with the YEp plasmid (Table 3). However, during the analysis of the number of colonies expressing β-galactosidase, different blue patterns were observed in the Petri plates, indicating the potential loss of gene copy number. This observation suggests that the long-term stability of the constructed strain in the nonselective continuous culture may be problematic. In fact, genetic instability is a major problem of prolonged continuous fermentations with recombinant microorganisms. For continuous cultures, further stability improvements may be achieved by changing culture conditions and operating strategies as described by others in regard to plasmid instability in S. cerevisiae continuous cultures (29).

In conclusion, the use of the δ-integration system together with G418 selection resulted in the construction of flocculent S. cerevisiae strains producing extracellular A. niger β-galactosidase at levels comparable to the multicopy YEp plasmid system in a batch culture. For the continuous high-cell-density culture, the integrant strain was more stable and presented a higher specific β-galactosidase activity than the plasmid-carrying strain, thus presenting important advantages over previously constructed strains.

ACKNOWLEDGMENTS

We thank Luis Lima for helping in the continuous-culture experiments. We thank Prof. Merja Penttilä, VTT-Biotechnology, Espoo, Finland for providing the pVK1.1 plasmid. Carla Oliveira was supported by the grant SFRH/BD/19099/2004 from Fundação para a Ciência e Tecnologia, Portugal.

REFERENCES


