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Evaluation of different promoters for the efficient production of heterologous proteins in baker's yeast

A. Monfort, S. Finger, P. Sanz & J.A. Prieto*
Departamento de Biotecnología, Instituto de Agroquímica y Tecnología de Alimentos (CSIC), P.O. Box 73, 46100 Burjassot (Valencia), Spain
**Author for correspondence (E-mail: prieto@iata.csic.es)*

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Abstract

We have constructed reporter gene expression vectors placing the *Aspergillus oryzae* α-amylase cDNA and the *Geotrichum candidum* lipase 2 gene, under the control of the yeast gene promoters,*ACT1*, *ADH1*, *PGK1*, and *TDH1*. Expression regulated by the shorted form of the *ADH1* promoter gave the highest proteins yield for cells cultured in molasses medium or flour/water mixtures. Nevertheless, the *ACT1* promoter appeared as the strongest in cells growing actively with sucrose.

Introduction

The expression of heterologous genes in *Saccharomyces cerevisiae* has open the possibility of using baker's yeast as an enzyme cell factory (for a review, see Randez-Gil *et al.* 1999). The significance of this strategy is two-fold. It avoids the use of crude enzyme preparations in the baking industry thus overcoming some drawbacks of this practice (Randez-Gil *et al.* 1999). In addition, it allows the recombinant enzyme to be recovered from the culture medium after the industrial propagation process. Based on this, new baker's yeast strains producing α-amylase (Randez-Gil *et al.* 1995, Monfort *et al.* 1996), endoxylanase (Monfort *et al.* 1996, 1997) or lipase (Monfort *et al.* 1999) have been constructed. In these approaches, the expression of foreign genes was placed under the control of the *ACT1* gene constitutive promoter (Tuite 1991). Although the use of those recombinant strains in bread making resulted in a positive effect on bread quality (Randez-Gil *et al.* 1999), the level of enzyme production was in some cases inadequate to obtain the overall expected benefit (Monfort *et al.* 1999).

A fundamental factor influencing the expression of heterologous genes in yeast is the level of transcription provided by the promoter (for a review, see Hadfield *et al.* 1993). Strong constitutive promoters have been therefore often chosen for efficient foreign protein production in *S. cerevisiae* (Nacken *et al.* 1996). Among them, the promoters most commonly used have been those of the glycolytic genes, phosphoglycerate kinase, *PGK1*, alcohol dehydrogenase, *ADH1* and glyceraldehyde-3-phosphate dehydrogenase, *TDH1* (Hadfield *et al.* 1993). Although those promoters are referred as constitutive, their transcriptional activity is subjected to regulation, reflecting a particular physiological state of the cell (Hadfield *et al.* 1993, Ruohonen *et al.* 1995). The choice of a promoter for heterologous gene constructions should reflect therefore the particular condition in which the protein production may be conducted.

Here, we have studied the limits of protein production levels by varying promoter strength in baker's yeast transformants grown in industrial media.

Materials and methods

Strains and culture media

Genomic DNA of the *Saccharomyces cerevisiae* ENY.WA-1A strain (*MAT*α, *ura 3-52*; *his3*-Δ1; *leu*

2-3, 112; *trp 1-289*; *MAL 2-8*^{c}, *MAL 3, SUC 3*) was used to amplify the different yeast promoters by PCR. The industrial baker's yeast strain CECT10837 (aneuploid, *trp1*) was used in transformation experiments (Randez-Gil & Sanz 1994). Yeast cells were grown in YNB (0.67% yeast nitrogen base) or in YP rich medium (1% yeast extract, 2% peptone) supplemented with nutrient requirements and glucose (2%) as carbon source. Cells were also grown in molasses medium as described in Randez-Gil *et al.* (1995). To simulate a real dough system, cells propagated in molasses medium were inoculated (2% wet weight, flour basis) in a non-sterile water/flour slurry (20 g of wheat flour, 0.4 g of NaCl, in 50 ml of water). Cultures were incubated by shaking (200 rev/min) at 30 °C in Erlenmeyer flasks. Starch-agar medium (Randez-Gil & Sanz 1994) was used to test the production of α-amylase. The oil-agar medium described by Baillargeon *et al.* (1989), was used to detect the production of recombinant lipase.

Escherichia coli DH10B strain was used as a host for plasmid constructions and was grown in Luria Bertani medium (1% peptone, 0.5% yeast extract, 0.5% NaCl).

Plasmids and cloning procedures

The functional part of the short *ADH1* (*ADH1*s), middle *ADH1* (*ADH1*m), *PGK1* and *TDH1* gene promoters were amplified by PCR from *Saccharomyces cerevisiae* genomic DNA. The *ADH1*s and *ADH1*m promoters (Ruohonen *et al.* 1995) were amplified using the oligonucleotides Adh1-1 (5′-GGGATAGACATTcTAgATGAGATAG-3′; +11/−14) and Adh1s-2 (5′-GTAATAATAGGCGgATcCAACTTC-3′; −426/−403) and Adh1m-2 (5′-GTTGTTTCCGGaTccACAATATGG-3′; −709/−686) respectively. The *PGK1* gene promoter (Ogden *et al.* 1986) was amplified using the oligonucleotides Pgk1-1 (5′-AGATTCCTGgaTcCAACTCAAGAC-3′; −772/−749) and Pgk1-2 (5′-GACAACTTTGAAGATctAGACtTTG-3′; +23/−2). The *TDH1* gene promoter (Bitter & Egan 1984) was amplified using the oligonucleotides Tdh1-1 (5′-ACCATATGGAGGATccGTTGGG-3′; −714/−693) and Tdh1-2 (5′-GTGTGTAAATcTAGaGAAGTACTG-3′; −9/−32). (The *Bam*HI and *Xba*I sites are underlined). The amplified fragments were subcloned into the pUC18-*Sma*I plasmid, resulting in plasmids pUC-pADH1s, pUC-pADH1m, pUC-pPGK1 and pUC-pTDH1. The YEpACT-AMY (Randez-Gil *et al.* 1995) and YEpACT-LIP2-t (Monfort *et al.* 1999) plasmids containing, respectively, the *A. oryzae* α-amylase cDNA and the *Geotrichum candidum* lipase 2 gene under the control of the *S. cerevisiae* actin (*ACT1*) gene promoter, were used to construct the reporter gene expression plasmids. The *ACT1* promoter was released from YEpACT-AMY and YEpACT-LIP2-t by digestion with *Bam*HI/*Xba*I and *Eco*RI/*Xba*I, respectively, and replaced with the *ADH1*s, *ADH1*m, *PGK1* and *TDH1* promoter fragments generated from the corresponding pUC18 constructs with the same set of enzymes. In this way we obtained the plasmids YEpADHs-AMY, YEpADHm-AMY, YEpPGK-AMY, YEpTDH-AMY, YEpADHs-LIP2-t, YEpADHm-LIP2-t, YEpPGK-LIP2-t and YEpTDH-LIP2-t (Figure 1).

Transformation protocols

Yeast transformation was carried out as described by Ito *et al.* (1983), using 3 μg plasmid DNA. *E. coli* was transformed by electroporation following the manufacturer's instructions (Eppendorf).

Enzyme determinations

α-Amylase activity was determined by the Ceralpha method (Sheehan & McCleary 1988), using blocked *p*-nitrophenyl maltoheptaoside as substrate. One α-amylase unit is defined as the amount of enzyme that is able to release 1 μmol *p*-nitrophenol per min at 40 °C under the assay conditions.

Lipase activity was determined by measuring the release 4-methylumbelliferone by the action of the enzyme on 4-methylumbelliferyl-oleate (Monfort *et al.* 1999). One unit of enzyme activity is defined as the amount of enzyme necessary to produce 1 μmol 4-methylumbelliferone per min at 45 °C under the assay conditions.

Results and discussion

Effect of different promoters on heterologous protein production in molasses medium

We have already described that expression in baker's yeast strains of the *A. oryzae* α-amylase cDNA and the *Geotrichum candidum* lipase 2 gene from high copy number plasmids, YEpACT-AMY and YEpACT-LIP2-t respectively, showed low clonal variation and that both gene product were easily measured in either liquid medium or plate assay (Randez-Gil *et al.* 1995,

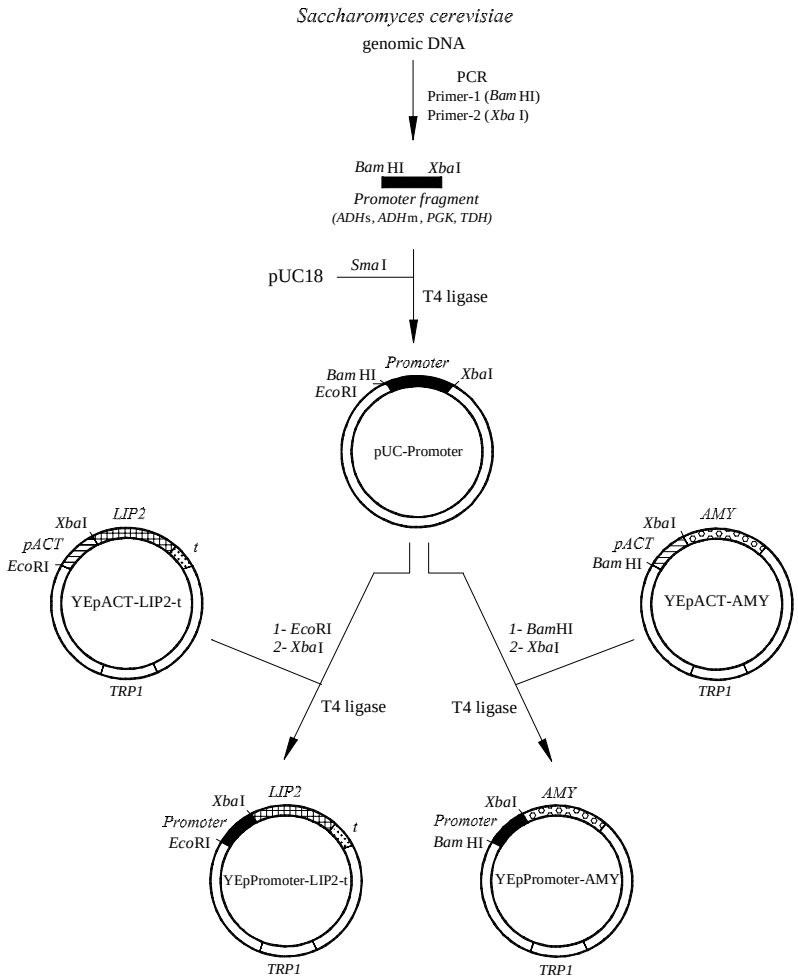

Fig. 1. Schematic representation of the construction of the YEpADHs-AMY, YEpADHm-AMY, YEpPGK-AMY, YEpTDH-AMY, YEpADHs-LIP2-t, YEpADHm-LIP2-t, YEpPGK-LIP2-t and YEpTDH-LIP2-t. Amplification by PCR was carried out with specific oligonucleotides. Details of the YEpACT-AMY and YEpACT-LIP2-t plasmids were already given (Randez-Gil *et al.* 1995, Monfort *et al.* 1999). The terminator of the *FBP* gene is present in all lipase expression vectors (t).

Monfort *et al.* 1999). We considered therefore that these genes were suitable as reporters for studying the heterologous protein production in baker's yeast by varying the promoter strength in the gene constructions. Highly reputed strong constitutive promoters, *ADH1*, *PGK1* and *TDH1*, were chosen to drive the expression of the two reporter genes using the *ACT1* promoter as control. Since several fragments of the original *ADH1* promoter have been characterised as differing in regulation (Ruohonen *et al.* 1995), it was decided to check the shorter form (*ADH1*s) and the middle form (*ADH1*m) in this study. Inducible promoters as those of the GAL system were discarded because a molasses medium, with sucrose as the predominant sugar, is commonly used in the commercial production of baker's yeast. In addition, application of recombinant yeast strains into the bread making system requires a high continuous enzyme production to obtain the desired effect (Randez-Gil *et al.* 1999).

Using the vectors, YEpACT-AMY and YEpACT-LIP2-t, we constructed various expression systems differing in the promoter just by exchanging the *ACT1* promoter DNA piece for that of the *ADH1*s, *ADH1*m, *PGK1* and *TDH1* promoters (Figure 1). The activity of the different constructs was analysed by measuring the production of the reporter protein at different growth stages on molasses medium. As it can be observed in Figure 2, lipase activity accumulated in the culture supernatant of all transformants analysed. The pattern of lipase production differed however be-

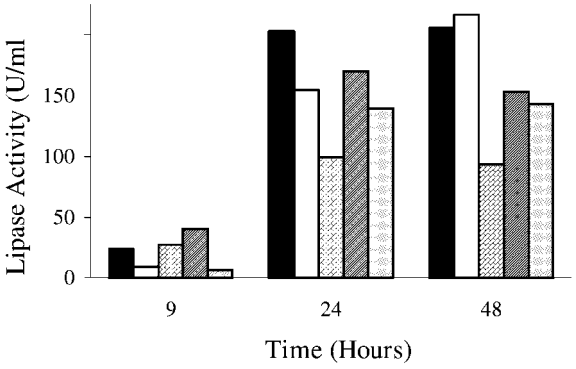

Fig. 2. Production of lipase activity at different stages of growth of baker's yeast transformants cultived in molasses medium. Plasmids contained in each strain, ■ YEpACT-LIP2-t, □ YEpADHs-LIP2-t, ▨ YEpADHm-LIP2-t, ▩ YEpPGK-LIP2-t and ▨ YEpTDH-LIP2-t, are indicated. Lipase was measured in the supernatant of the corresponding cultures.

tween cells containing expression cassettes controlled by *ACT1*, *ADH1*s, *ADH1*m, *PGK1* or *TDH1* promoters. At early exponential growth phase (cell dry weight approx. 1.5 mg/ml), lipase activity obtained from the *PGK1*p construct reached the highest value (40 U/ml), followed by those of *ADH1*mp (27 U/ml) and *ACT1*p (24 U/ml). This picture changed after 24 h of growth, when cells entered the ethanol consumption phase (cell dry weight approx. 7.8 mg/ml). At this stage, production of lipase by YEpACT-LIP2-t cells appeared as the strongest (203 U/ml), whereas the lipase activity under the control of the *ADH1*m promoter reached the poorest level, around twofold lower. This is an interesting observation because the *ACT1* promoter had been traditionally defined as a weak constitutive promoter (Tuite 1991). In our hands, this does not appear to be the case for baker's yeast growing actively in molasses medium. All constructs turned off during growth on ethanol (from 24 h to 48 h), except the short *ADH1* promoter which remained active. This result is in agreement with previous reports showing the expression pattern of this shortened form of the *ADH1* promoter in rich glucose medium (Ruohonen *et al.* 1995). The final higher yields corresponded to those constructions under the control of the *ACT1*p or the *ADH1*sp. Similar kinetics were observed with α-amylase as the reporter protein (data not shown). These results were not the consequence of differences in the level of plasmid loose, that was estimated in around 50% for all of them after 48 h of growth in molasses medium.

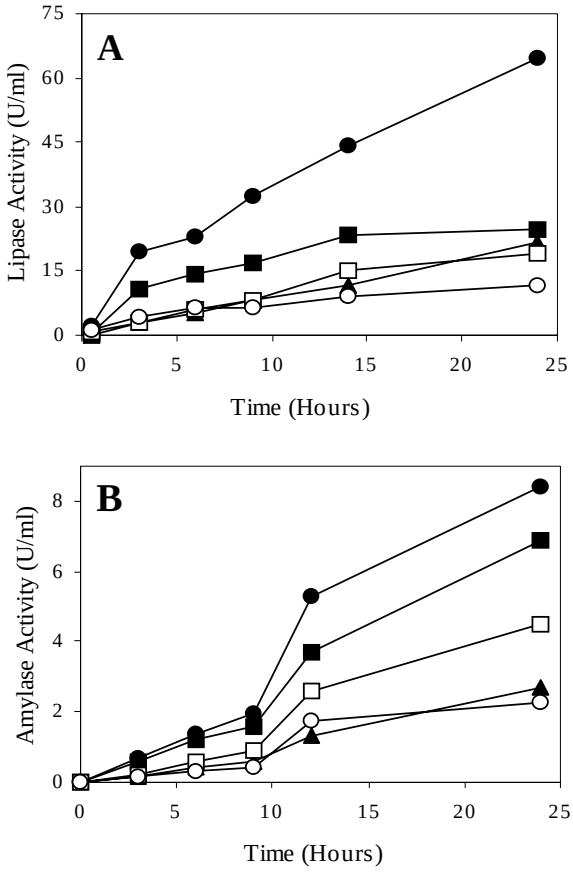

Fig. 3. Lipase (A) and α-amylase (B) activities along the culture of different baker's yeast transformants in flour/water mixtures. Plasmids containing lipase 2 and α-amylase reporter genes ■ YEpACT-, ● YEpADHs-, ▲ YEpADHm-, □ YEpPGK- and ○ YEpTDH-, were used to transform the industrial baker's yeast strain CECT10837. Aliquots of flour/water mixtures were centrifuged at 20, 800 × *g* for 10 min, and the levels of the two enzymes measured in the supernatant.

Evolution of reporter protein levels during yeast culture in flour/water mixtures

Heterologous protein production levels were also determined in baker's yeast cells cultured in a flour/water slurry, a medium that mimics the dough system. Direct assays in bread dough were ruled out because the developing of the gluten network during the mixing step generates protein-lipid aggregates that make more difficult the enzyme extraction, especially that of lipase.

Figure 3 shows the kinetic of α-amylase and lipase activity production found in the culture supernatant of YEpACT-, YEpADHs-, YEpADHm-, YEpPGK- and YEpTDH- transformants. All constructs produced the reporter proteins in the flour/water mixture but

with varying yields. Again, constructions where the reporter gene was under the control of the *ADH1*s promoter gave the best results, followed by those driven by the *ACT1* promoter. Differences were more pronounced at long fermentation times, 5 h or more, specially for lipase production (Figure 3B). At this time, the free sugars initially present in flour, mainly glucose, fructose, sucrose and glucofructans have been already consumed (Beudeker *et al*. 1990). This could explain the behaviour of *ADH1*sp constructs, since gene expression under the regulation of this promoter becomes significant only when glucose has been exhausted (Ruohonen *et al.* 1995).

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