

Stable High-Copy-Number Integration of *Aspergillus oryzae* α -AMYLASE cDNA in an Industrial Baker's Yeast Strain

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The *Aspergillus oryzae* α -amylase cDNA was placed under the control of the *Saccharomyces cerevisiae* actin promoter (*pACT1*) and introduced into the ribosomal DNA locus of an industrial baker's yeast strain. To obtain a strain eligible for commercial use, we constructed an integrative cassette lacking bacterial DNA sequences but containing the α -amylase cDNA and ribosomal DNA sequences to target the integration to this locus. High-copy-number integrants were obtained including a defective *TRP1d* promoter in the integrative cassette. We selected one transformant, Rib-AMY (CECT10872), in which the multi-integrated sequences were stable even after 200 generations of growth in nonselective medium. This transformant also expressed and secreted high levels of α -amylase. Bread made with this strain had a higher volume, lower density, and softer crumbs than bread made with a control strain. The Rib-AMY transformant also was useful in retarding bread firming. This new strain fulfills all the requirements for commercial utilization and should reduce or eliminate the requirement for addition of exogenous α -amylase to the flour, reducing allergenic work-related symptoms due to this enzyme.

Introduction

Aspergillus oryzae α -amylase is widely used as a baking additive. This enzyme improves yeast growth and gas production (1, 2). In addition, it delays the rate of staling and increases bread shelf life (1–4). The main source of this additive is the concentrated liquid culture medium of *A. oryzae*. These preparations may contain other enzymes secreted by the fungus that reduce bread quality (1, 2). On top of this, the incidence of allergenic symptoms in bakers after using the α -amylase as a crude enzyme is very well documented (5, 7).

We expressed *A. oryzae* α -amylase cDNA in commercial baker's yeast strains under the control of the *Saccharomyces cerevisiae* actin promoter (*pACT1*), which allows proper expression of this gene in standard bread-making procedures. These recombinant strains have been used to make bread and produced bread of better quality with higher volume, lower density, and softer crumbs than bread made with a control strain (8). Unfortunately, the vector used in that work did not fulfill two main criteria for a commercially useful genetically modified organism: (i) absence of vector sequences related to prokaryotic DNAs or antibiotic resistance markers and (ii) high mitotic stability of the foreign gene under nonselective conditions. The YEp derivative plasmid used in ref 8 contained prokaryotic sequences and the *Amp^R* resistance marker and was not mitotically stable.

In *S. cerevisiae*, the usual way to meet these requirements is to introduce the foreign DNA into a yeast chromosome by homologous recombination of heterologous gene attached to a yeast selection marker (9). The foreign sequences are usually stably maintained at low

copy number. To produce high levels of heterologous proteins usually requires a high copy number of the expression cassette. For example, in our previous work (8), the production of enough *A. oryzae* α -amylase to affect bread quality and firming was correlated with the presence of multiple copies of the fungal α -amylase cDNA in an episomal plasmid (YEp), which may be present at up to 100 copies per cell (10).

One approach to increase the copy number of chromosomally inserted genes is to target integration into the ribosomal DNA (rDNA) locus and select for multiple integration using defective selection markers such as *LEU2d*, *URA3d*, or *TRP1d* (11–13). Our objectives were to construct a set of integrative cassettes that contained the *A. oryzae* α -amylase gene with different *TRP1d* defective markers to promote multiple integration, flanked by ribosomal sequences to target the integration into the ribosomal rDNA locus.

Materials and Methods

Strains and Culture Conditions. *Escherichia coli* DH10B was used for plasmid constructions (14). The industrial baker's yeast strain CECT10837 [*trp1* (15)] and the laboratory strain ENY.WA-1A [*MAT α* , *ura3-52*, *leu2-3,112*, *trp1-289*, *his3- Δ 1*; (16)] were used in transformation experiments. The commercial baker's yeast strain named Cinta Roja was used as a control in the bread-making tests.

Yeast cells were grown in rich medium YPD (1% yeast extract, 2% peptone, 2% glucose) or in minimal medium MM [0.67% yeast nitrogen base without amino acids from (Difco)] containing 2% glucose (MMG) or in combination with 1% soluble starch (MMS). Yeast biomass was prepared by growing cells in a liquid molasses medium according to an industrial recipe: 116 g of beet molasses (49% sucrose), 1.5 g of ammonium sulfate, 0.15 g of

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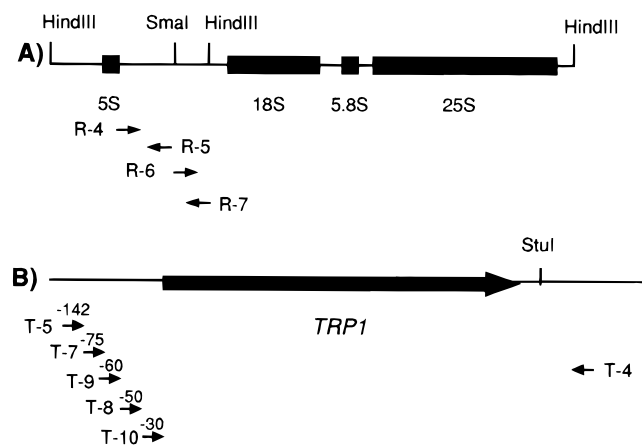


Figure 1. (A) Diagram of the tandemly repeated ribosomal rDNA locus unit. (B) Diagram of the *TRP1* locus. The positions of the different oligonucleotides used in the PCR reactions are indicated.

orthophosphoric acid, and 0.1 mg of biotin per liter at pH 5.0. Cultures were grown in batch and incubated at 30 °C in agitation (200 rpm) until they reached an A_{600} of about 15 (using a Spectronic 20 spectrophotometer, Milton Roy, Rochester, NY); cells were then collected by centrifugation and washed three times in cold (4 °C) water. The resulting cake was filtered through a porous plate (Pyrex no. 5, England) to remove the excess of water and kept at 4 °C for later use. In all cases, storage time was no longer than 24 h.

Construction of Plasmids. All DNA manipulations were performed according to standard protocols (17). PCR reactions were carried out following a general protocol (18): 30 cycles (95 °C, 30 s; 56 °C, 30 s; 72 °C, 2 min) followed by an extension of 15 min at 72 °C.

Ribosomal sequences to target DNA recombination were amplified using two pairs of oligonucleotides R4-R5 and R6-R7 and *S. cerevisiae* genomic DNA as template: R4, 5'-TATTGAGCTCATGAGAGTAGC-3' (*SacI* site underlined); R5, 5'-GTGACAGGTACCCCGGGTAA-3' (*KpnI* site underlined); R6, 5'-GTTACCCGGGCACCTGTC-3' (*SmaI* site underlined); R7, 5'-TTTCCACTAGTCAGGTTCCAC-3' (*SpeI* site underlined). The amplified ribosomal fragments corresponded to NTS sequences between the 5S and 18S subunits (Figure 1-A). The fragment R4-R5 was subcloned in YEACT-AMY (8) after digestion with *SacI* and *KpnI*, resulting in plasmid YEPR45-ACT-AMY (Figure 2). The fragment R6-R7 was digested with *SmaI* and *SpeI* and subcloned in the pBlueScript plasmid, resulting in plasmid pBS-R67 (Figure 2).

TRP1d defective markers were amplified by PCR using several pairs of oligonucleotides (T5-T4, T7-T4, T8-T4, T9-T4, and T10-T4): T4, 5'-GCGGTGAAATGGTAAAGTCAACC-3'; T5, 5'-GCTCGAGGGGCCGCATTAAAGCTTATCGATGAT-3' (-142 bp to ATG; *XhoI* and *ApaI* sites underlined); T7, 5'-GAAACTCGAGGGCCAAGAGGGAG-3' (-75 bp to ATG; *XhoI* site underlined); T8, 5'-TGACTCTCGAGCACGTGAGTATACG-3' (-50 bp to ATG; *XhoI* site underlined); T9, 5'-GGGAGGGCCCTGGTGAC-TATTG-3' (-60 bp to ATG; *ApaI* site underlined); T10, 5'-GTGAGGGCCCGTGATTAAGCACACAA-3' (-30 bp to ATG; *ApaI* site underlined) (Figure 1B). The different *TRP1d* amplified sequences were subcloned into the *EcoRV* site of pBlueScript. Initially, the T5-T4 fragment containing a 142 bp *TRP1* promoter was used in the constructions, resulting in plasmids pBST-142-A and pBST-142-B (Figure 2). When shorter forms of the

promoter were needed, either a *XhoI*-*StuI* or an *ApaI*-*StuI* fragment of the corresponding constructs was exchanged with other *XhoI*-*StuI* (T7-T4 and T8-T4) or other *ApaI*-*StuI* (T9-T4 and T10-T4) fragments containing shorter forms of the promoter (75 bp in T7-T4, 50 bp in T8-T4, 60 bp in T9-T4, and 30 bp in T10-T4).

To get the best expression of both the *TRP1d* and α -amylase cDNA, we made three different constructions named as series A, B, and C. Series A and B contained the *TRP1d* marker in front of the *pACT1-AMY* cassette in the two possible orientations. They were constructed as follows: (i) plasmids pBST-142-A and pBST-142-B (Figure 2) were digested with *KpnI* and *BamHI* to release a fragment that was ligated into YEPR45-ACT-AMY (see above), and the resulting plasmids were named YEPR45-TA-ACT-AMY and YEPR45-TB-ACT-AMY (Figure 2); (ii) a *KpnI*-*HindIII* fragment containing the *pACT-AMY* sequence present in plasmid pUC-ACT-AMY (8) was introduced in plasmid pBS-R67 digested with the same set of enzymes, resulting in plasmid pBS-ACT-AMY-R67 (Figure 2); (iii) to get a fragment flanked with appropriate restriction sites, a *BamHI*-*SpeI* fragment from pBS-ACT-AMY-R67 was subcloned in pT7Blue, leading to plasmid pT7-ACT-AMY-R67 (Figure 2); (iv) finally, plasmids YEPR45-TA-ACT-AMY and YEPR45-TB-ACT-AMY were digested with *SacI* and *BamHI* and the resulting fragments were subcloned in pT7-ACT-AMY-R67 to obtain the pRT-A-AMY and pRT-B-AMY plasmids.

Series C contained the *TRP1d* marker downstream the *pACT-AMY* cassette (Figure 3). They were constructed as follows: (i) plasmid pBST-142-B (see above) was digested with *XhoI* and *SmaI* and the released fragment subcloned into pBS-R67, resulting in plasmid pBST-R67 (Figure 3); (ii) to get a fragment flanked with appropriate restriction sites, a *KpnI*-*SpeI* fragment from pBST-R67 was subcloned in pT7-Blue, leading to plasmid pT7T-R67 (Figure 3); (iii) plasmid YEPR45-ACT-AMY (see above) was digested with *SacI* and *HindIII* and the released fragment subcloned in pBlue-Script, resulting in plasmid pBSR45-ACT-AMY (Figure 3); (iv) finally, a *SacI*-*XhoI* fragment from plasmid pBSR45-ACT-AMY was subcloned into pT7T-R67, resulting in plasmid pRAMY-T-C (Figure 3).

In the series C, some of the representatives contained the *FBP1* (fructose biphosphatase) gene terminator between the *pACT-AMY* cassette and the *TRP1d* marker. The insertion of this terminator was carried out as follows: (i) the *FBP1* terminator was amplified by PCR using oligonucleotides FBP-1 (5'-GGTACTCGAGGATCGCCTTCTTTTC-3', *XhoI* site underlined) and FBP-2 (5'-CTTTTCGGGGCCCGTAGTACAATTC-3', *ApaI* site underlined); (ii) plasmid pR-AMY-T-C was digested with *XhoI* and *ApaI* to introduce the *FBP1* terminator digested with the same set of enzymes, generating plasmid pRAMY-t-T-C (Figure 3).

Construction of YEp-Multicopy Control Plasmids. To test α -amylase and *TRP1* expression in multiple copies, plasmids pRT-A-AMY, pRT-B-AMY, pRAMY-T-C, and pRAMY-t-T-C were digested with *SpeI* and overhanging ends filled with Klenow; then, they were cut with *SacI* to release the corresponding "integrative cassettes" containing the α -amylase cDNA, the *TRP1d* selection marker, and the ribosomal sequences and ligated into YEplac181 (19) digested with *SacI* and *SmaI*.

Yeast Transformation. pRT-A-AMY, pRT-B-AMY, pRAMY-T-C, and pRAMY-t-T-C (10 μ g each) were digested with *SacI* and *SpeI* to release their corresponding "integrative cassettes" (Figures 2 and 3). These fragments were gel purified and used for integrative yeast trans-

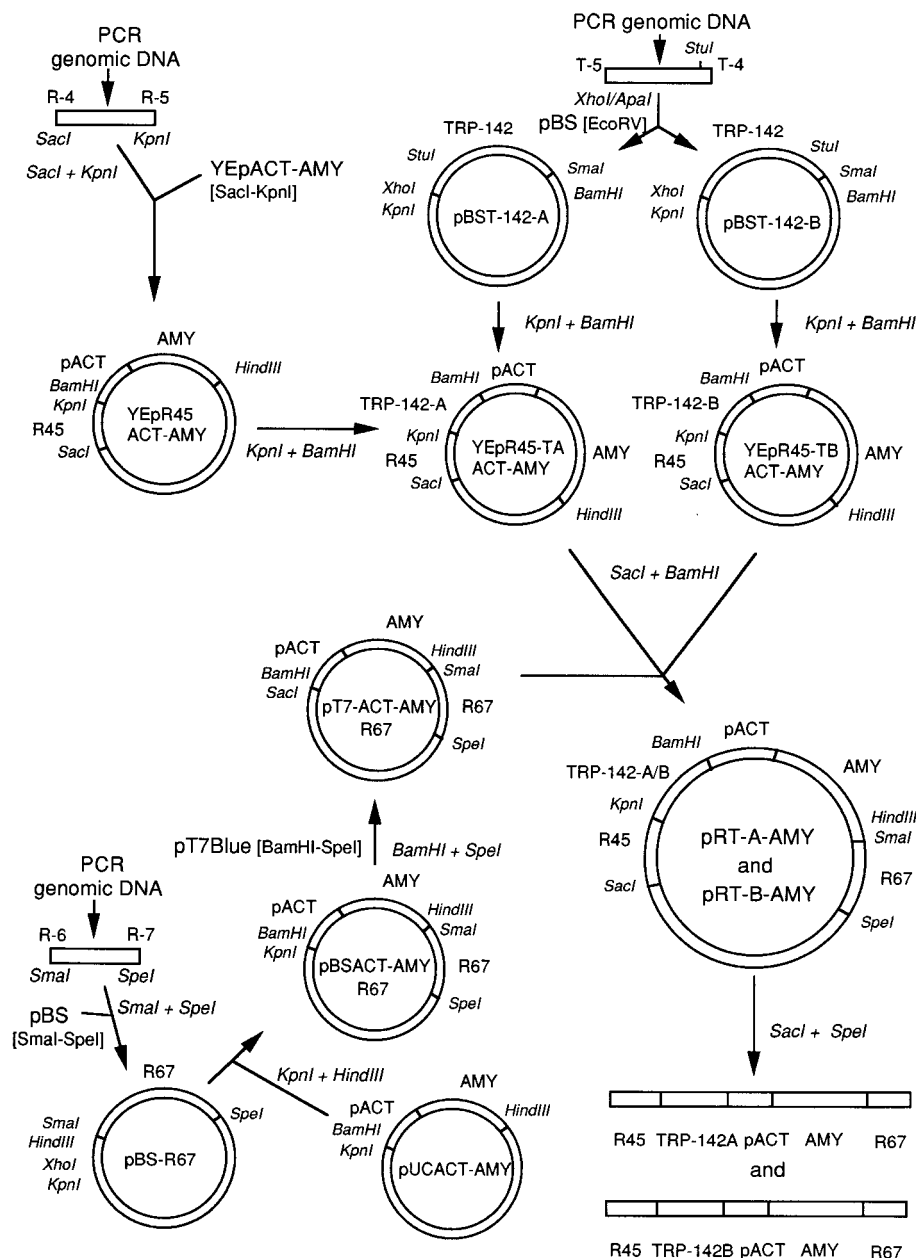


Figure 2. Construction of pRT-A-AMY and pRT-B-AMY plasmids. See the Materials and Methods for details.

formations by following the lithium acetate protocol (20) and using the CECT10837 baker's yeast strain as a host. The same plasmids were digested with *StuI* to target integration of the whole vector to the *TRP1* locus. In this way, we obtained transformants with only one copy of the "integrative cassette" inserted into the yeast chromosome, which were used as control of integration in single copy.

The multicopy control plasmids (YE_p derivatives) constructed above were used to transform the laboratory strain ENY.WA-1A of *S. cerevisiae*. In this way we obtained control transformants that carried multiple copies of the vector.

α -Amylase Determination. α -Amylase activity was determined by the Ceralpha method (21) using blocked *p*-nitrophenyl maltoheptaoside as substrate. One α -amylase unit is defined as the amount of enzyme that is able to release 1 μ mol of *p*-nitrophenol/min at 40 °C under the assay conditions.

To determine this enzyme in fermented doughs, 25 g of dough was homogenized in extraction buffer (50 mM

sodium malate, 50 mM NaCl, 2 mM CaCl₂, 0.005% Na N₃, pH 5.2) as previously described (8). The mixtures were centrifuged at 10000*g* for 10 min, and the α -amylase was measured in the supernatant, as detailed above.

Mitotic Stability Determination. High-copy-number integrative transformants were cultured in 5 mL of YPD rich medium. When the cultures reach an *A*₆₀₀ between 4 and 6 units/mL, they were successively diluted to an *A*₆₀₀ of 0.001 units/mL to keep exponential growth. Cell samples were collected after 30, 100, and 200 generations and plated for single colonies on YPD, then checked for loss of markers by replica plating on MMS medium containing or lacking tryptophan. Two hundred colonies were analyzed at each time point. α -Amylase activity was tested by formation of starch haloes of hydrolysis when the plates were exposed to iodine vapors.

Genomic DNA Purification and Southern Blot Hybridization. Total yeast genomic DNA was purified as described previously (22). Probes for Southern blot hybridization analysis were labeled with [α -³²P]dCTP using a Ready-To-Go kit (Pharmacia). Hybridizations

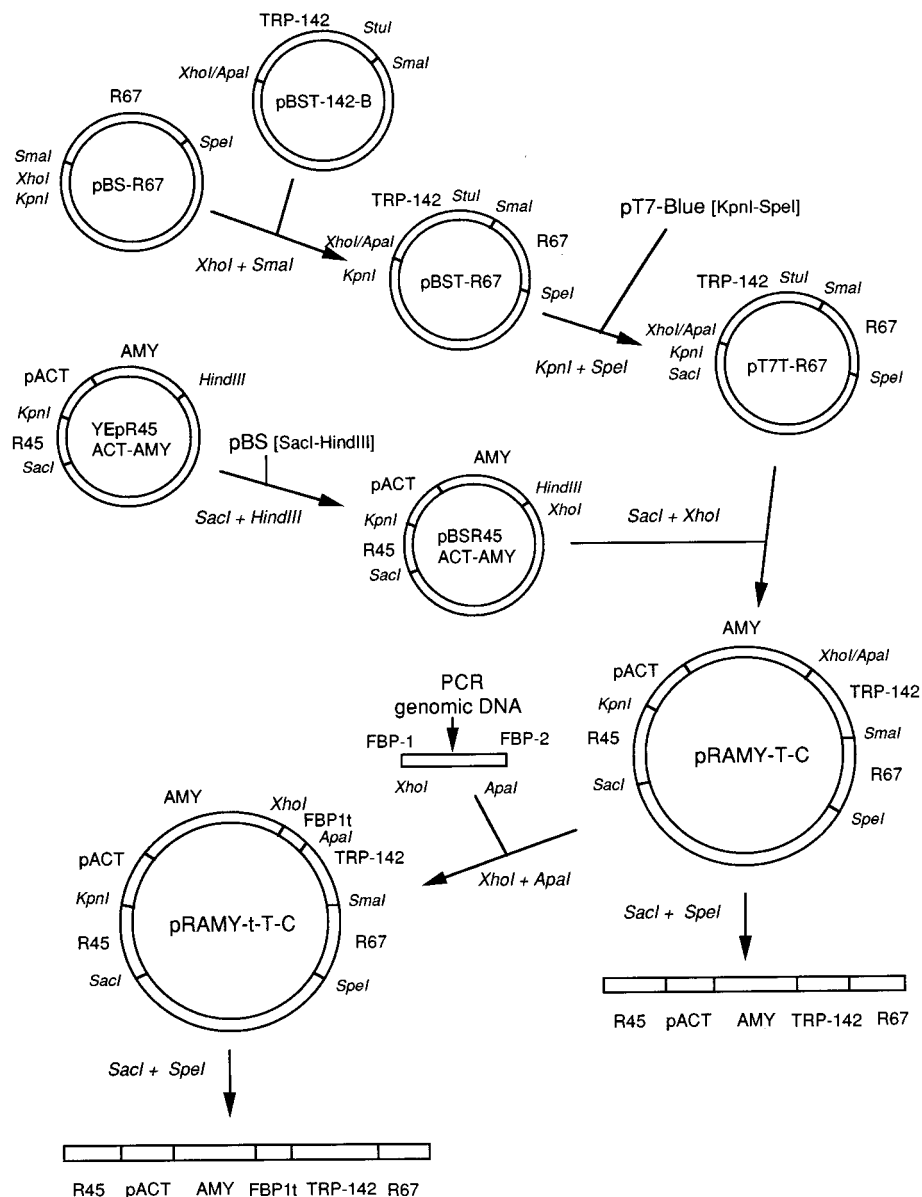


Figure 3. Construction of pRAMY-T-C plasmid and derivatives. See the Materials and Methods for details.

were carried out according to standard protocols using 50% formamide (17).

Bread-Making Procedure. A commercial wheat bread flour [moisture 13.5%; W, 14×10^{-3} J (Alveograph); P/L, 0.33; Falling number, 339 s] was used through the work. Bread dough was prepared by the straight dough process (23). Bulk fermentation and proofing were performed in a proofing cabinet (28 °C, 80% relative humidity). Bread was stored for 5 days in polyethylene bags at 4 °C or room temperature. The baking test was carried out at least in duplicate.

Bread Volume and Crumb Texture Assessment. Loaf volumes measurements were taken 2 h after baking by the rapeseed displacement procedure. At least six pieces of bread were measured for each yeast. Bread crumb texture was determined as the maximum deformation strength in a 2-cm-thick slice on an Instron model 1140 press (Instron food testing machine), equipped with a 17-mm-diameter probe. Bread was automatically sliced (ROLS, Barcelona, Spain), and deformation strength values were registered from the central slices, measuring the force required to compress each slice to 25% of its

original thickness. At least six loaves were assayed for each yeast and storage time.

Statistical Treatment. One-way analysis of variance (ANOVA) was performed with an A IBM AT computer (BMDP statistical package, 7D program). The least significant differences among means were obtained after applying the Q test.

Results

Construction of Integrative Cassettes for α -Amylase Expression. To obtain multiple-copy stable integration of the *A. oryzae* α -amylase into an industrial baker's yeast strain, we designed different series of constructions where the α -amylase gene and the *TRP1d* marker were flanked by ribosomal sequences.

Series A and B contained the *TRP1d* marker in front of the *pACT-AMY* cassette, in the two possible orientations (pRT-A-AMY and pRT-B-AMY, Figure 2). Series C contained the *TRP1d* marker downstream the *pACT-AMY* cassette (pRAMY-T-C, Figure 3). The *TRP1d* marker present in all these plasmids contained the complete ORF and a promoter of 142 bp in length. These plasmids were

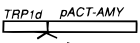
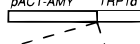
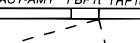
	TRANSFORMATION					
	Integrative				Non-integrative	
	<i>TRP1</i> locus (<i>StuI</i> digestion)		Ribosomal locus (<i>SacI-SpeI</i> digestion)		YEp-multicopy derivatives	
	<i>TRP</i> ⁺	<i>AMY</i> ⁺	<i>TRP</i> ⁺	<i>AMY</i> ⁺	<i>TRP</i> ⁺	<i>AMY</i> ⁺
A Series						
	-	-	-	-	-	+++
142 bp						
B Series						
	+	+	+	+	+	+++
142 bp						
75 bp	+	+	+	+	+	+++
50 bp	-	-	+	+++	+	+++
30 bp	-	-	-	-	-	+++
C Series						
	-	-	-	-	-	+++
142 bp						
	+	+	+	+	+	+++
142 bp						
60 bp	+	+	+	+	+	+++
30 bp	+	+	+	+	+	+++

Figure 4. Tryptophan prototrophy and α -amylase activity detected in different transformants. All of the integrative transformations were performed in the industrial baker's yeast CECT10837 strain. Transformation with the YEp-multicopy control plasmids was carried out in the *S. cerevisiae* ENY.WA-1A strain. Tryptophan prototrophy: (+) growth, (-) no growth in the absence of tryptophan. α -Amylase activity: small (+), medium (++), and large (+++) starch haloes of hydrolysis in MMS medium after 24 h of growth.

digested with *SacI* and *SpeI* to release their corresponding "integrative cassette" (α -amylase cDNA, the *TRP1d* selection marker and the ribosomal sequences; see the Materials and Methods) that were used to transform the industrial baker's yeast strain CECT10837.

As it has been already described, the appearance of multiple-copy integrants is related to the use of a defective selection marker that allows the recovery of transformants only when the marker is present in multiple copies (8–10). In this way, if a single integration occurs, the defective marker is too weak to allow growth. Only when cells have multiple copies of it do they produce enough protein to allow them to grow under the selective conditions. To test the defectiveness of our *TRP1d* marker, we linearized the plasmids with *StuI* to promote the monocopy integration of the vector at the *TRP1* locus; we used these transformants as controls of monointegration. We also constructed YEp-derivative plasmids containing the "integrative cassettes" (see the Materials and Methods); transformants containing these plasmids were used as controls of the presence of the "integrative cassette" in multiple copy. In this latter case, *S. cerevisiae* ENY.WA-1A was used as a host and transformants were recovered using the selection marker present in the plasmid backbone (*LEU2*).

As shown in Figure 4, we were not successful in obtaining multicopy integrants when we used the "integrative cassette" of the A series, perhaps because the orientation of the *TRP1d* marker did not allow its appropriate expression. This was confirmed when we used the corresponding YEp-multicopy control plasmid

derivative: *LEU2*⁺ transformants were unable to grow in the absence of tryptophan.

When we used the "integrative cassette" of the B series we obtained only monocopy integrants. It seemed that the *TRP1d* marker was functional even when present in single copy (see *StuI* control of monointegration in Figure 4). To obtain a real defective marker we reduced the size of the promoter (see the Materials and Methods). We constructed in this way plasmids pRT-B75-AMY, pRT-B50-AMY, and pRT-B30-AMY that contained 75, 50, and 30 bp of the *TRP1d* promoter, respectively. As shown in Figure 4, only pRT-B50-AMY was able to produce multicopy integrants: the *TRP1-50d* marker was functional when present in multiple copies (see the corresponding YEp-multicopy control plasmid) but not when it was present in single copy (see *StuI* control of monointegration). In the other cases, the *TRP1-75d* marker was still functional in monocopy and the *TRP1-30d* marker was not functional even in multicopy.

When we used the "integrative cassette" of the C series, we were not able to obtain multicopy integrants, since the *TRP1d* marker was not functional, even when present in multiple copies (see YEp-multicopy control plasmid, Figure 4). One possible explanation was that the transcription of the *pACT-AMY* cassette interfered with the transcription of the *TRP1d* marker because of the close proximity of the stop codon of the *AMY* gene and the *TRP1d* promoter (only 60 bp). So we decided to include a yeast strong terminator (*FBP1t*) between the *pACT-AMY* cassette and the *TRP1d* marker, generating plasmid pRAMY-t-T-C (see the Materials and Methods). In this case, the *TRP1d* marker turned out to be functional even in single copy (Figure 4). To obtain a real defective marker we reduced the size of the promoter present. We constructed in this way plasmids pRAMY-t-T60-C and pRAMY-t-T30-C (see the Materials and Methods). Unfortunately, we did not obtain multicopy integrants in any of the cases, as the two markers were still functional when present in single copy (Figure 4).

In conclusion, only the "integrative cassette" present in plasmid pRT-B50-AMY was able to produce multicopy integrants. For this reason we used only this "integrative cassette" in further experiments.

Stability of Multicopy Integrants. The aim of the work was to construct a baker's yeast strain that stably maintained a high copy number of the α -amylase gene. To test whether the transformants obtained with the "integrative cassette" of the pRT-B-50-AMY plasmid were stably maintained, we grew them in a rich medium for up to 200 generations. Then, we checked the number of colonies retaining tryptophan prototrophy and amylolytic activity. After 200 generations in nonselective medium, 97% of the colonies of one transformant that we called Rib-AMY (CECT10872) were still prototroph to tryptophan and able to secrete α -amylase.

Integration of the α -Amylase Gene at the Ribosomal DNA Locus. Physical analysis of DNA isolated from the Rib-AMY multi-integrative transformant was carried out to analyze whether the integration had occurred at the expected locus and also to check whether this transformant was free of any vector sequence. Southern blot hybridization using the R4-R5 ribosomal fragment as a probe showed that the "integrative cassette" was integrated at the ribosomal locus as expected (Figure 5A,C). After the integration, a new 1.5 kb *EcoRI* band and a new 2.1 kb *HindIII* band appeared, which were in agreement with the size of the calculated bands. The extra band seen in the *EcoRI* digestion of the nontransformed strain may be due to polymorphisms of

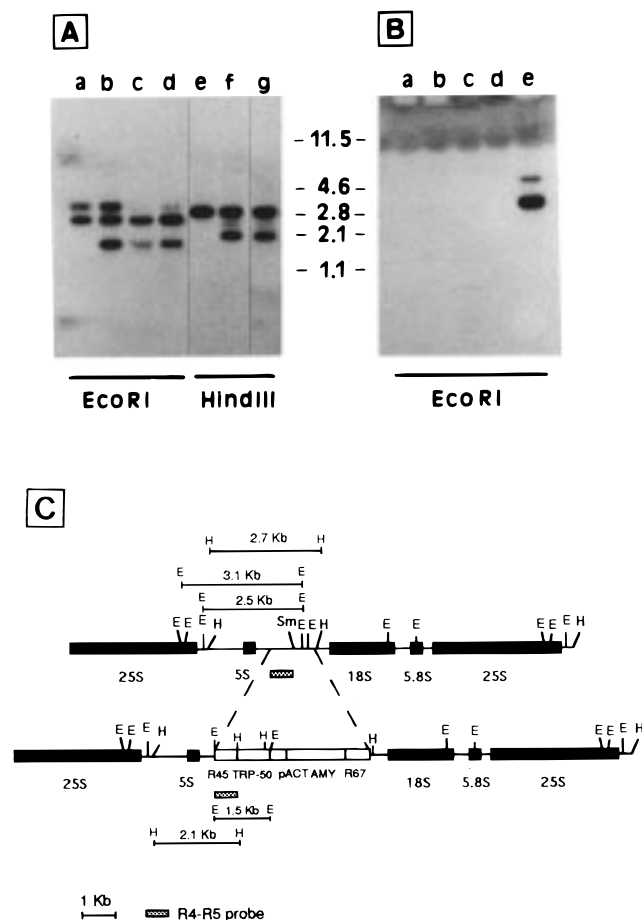


Figure 5. Analysis of the location of the "integrative cassettes" in the Rib-AMY transformant chromosome. (A) Southern blot hybridization using the ribosomal fragment R4-R5 as a probe: lanes (a-d), *EcoRI* digestion; lanes (e-g), *HindIII* digestion. Untransformed strain (lanes a and e), Rib-AMY transformant grown in YPD after 10 (lanes b and f), 30 (lane c), and 100 generations (lanes d and g). (B) Southern blot hybridization using a *ScaI-SspI* fragment (324 bp) from pUC containing the ampicillin resistance gene as a probe: *EcoRI* digestion of genomic DNA corresponding to untransformed strain (lane a), Rib-AMY transformant grown in YPD after 10 (lane b), 30 (lane c), and 100 generations (lane d), and a transformant carrying the entire pRT-B-50-AMY plasmid as positive control (lane e). (C) Diagram of the tandemly repeated ribosomal rDNA locus unit. Restriction sites are indicated as follows: E, *EcoRI*; H, *HindIII*; Sm, *SmaI*. Predicted integration of the "integrative cassette" is shown. Expected fragment sizes generated by *EcoRI* or *HindIII* digestions are indicated.

the rDNA locus in the strain used. When a fragment of pUC containing the *Amp^R* marker was used as a probe, no signal was obtained in the Rib-AMY transformant (Figure 5B), so it was free from prokaryotic sequences.

Copy number of the "integrative cassette" was calculated by comparison of the intensity of the new band with the intensity of the ribosomal band present in the wild-type strain (*HindIII* digestion). The intensity of the bands was measured using an LKB densitometer. We estimated around 50–100 copies per cell, as the new band (2.1 kb) had almost half intensity of the original one (2.7 kb), and it is assumed that cells contain 100–200 copies of the rDNA operon.

α -Amylase Production in Molasses Medium. The growth rate and the α -amylase production of the Rib-AMY transformant were compared with those of a commercial control strain. As shown in Figure 6, no differences in the growth rate were observed between both strains. However, the Rib-AMY transformant was

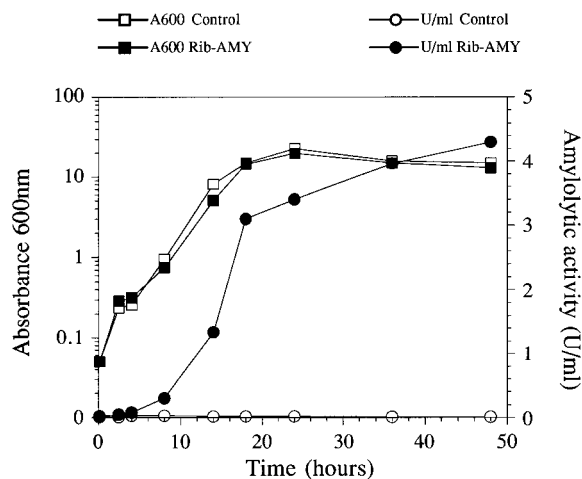


Figure 6. Growth curves and α -amylase production in molasses medium. The strains, commercial control and Rib-AMY, were inoculated at a starting A_{600} of 0.05 U/mL. At different times, the absorbance at 600 nm was determined and the α -amylase activity was measured in the supernatant.

able to secrete large amounts of α -amylase to the medium. The amount of α -amylase produced by the Rib-AMY transformant was even higher than the one produced by the [YEpACT-AMY] strain (8) grown under the same conditions (2.6 U/mg of cells dry weight versus 1.8 U/mg of cells dry weight, after 16 h of growth in molasses medium).

Application of the Rib-AMY Transformant in Bread Making. To test if the Rib-AMY transformant was suitable for bread making, we prepared with it bread doughs by the straight dough process. As shown in Table 1, the Rib-AMY transformant was able to produce α -amylase in fermented doughs. To test whether the amount of α -amylase produced was enough to improve bread quality, we prepared bread with these fermented doughs. Breads elaborated with the Rib-AMY transformant showed an increase in volume of about 8% and lower density than those prepared with the commercial control or the untransformed host yeast strain (Table 1). Crumb firming was also improved in breads made with the Rib-AMY transformant (Figure 7). After 5 days of storage at room temperature, the crumb firmness values were around 50% lower in breads elaborated with the Rib-AMY transformant versus the commercial control strain. Similar results were found for samples stored at 4 °C (data not shown).

Discussion

It is generally admitted that genetically modified organisms have to fulfill two main criteria for its commercial distribution: (i) absence of vector sequences related to prokaryotic DNAs or antibiotic resistance markers and (ii) high mitotic stability of the foreign gene in nonselective conditions. To achieve the first criteria, the fragments used in the transformations should be reduced to a minimum and contain only the information required for the modification. This imposition makes the genes contained in the transformation module very close to each other, and this may interfere with their expression. So different arrangements of the genes should be tested to see which one is the most effective. In our case we tested the combined expression of the *A. oryzae* α -amylase cDNA (AMY) controlled by the *S. cerevisiae* actin promoter (*pACT1*) and a *S. cerevisiae* *TRP1d* defective marker. We observed that the rate of the expression of the genes varied depending on the arrange-

Table 1. Levels of α -Amylase in Fermented Doughs and Volume and Density of Breads Elaborated with the Rib-AMY Transformant (CECT10872), the Untransformed Control Strain (CECT10837), and the Commercial Yeast Strain

strain	α -amylase (mU/g of fermented dough)	bread ^a	
		volume (mL)	density (g/mL)
commercial strain	56	345 $\pm$ 12	0.285 $\pm$ 0.011
CECT10837	60	340 $\pm$ 13	0.287 $\pm$ 0.010
Rib-AMY	590	373 $\pm$ 10 ^b	0.265 $\pm$ 0.010 ^b

^a Data represent the mean values ($\pm$ standard errors) of at least six pieces of bread in each baking test. ^b Indicates a statistically meaningful change between mean values ($P < 0.05\%$).

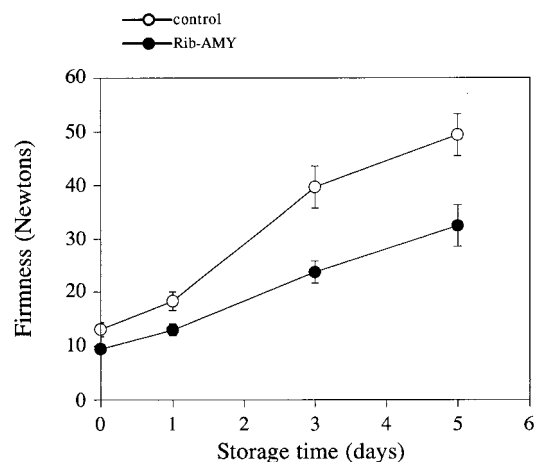


Figure 7. Firming curves of bread elaborated with different strains and stored at room temperature (25 °C). Bread was baked from fermented doughs prepared with a commercial control or Rib-AMY yeast strains. Bars represent standard errors of means. At least six measurements in each of two independent baking test were carried out for each sample.

ment of the corresponding promoters. When the *TRP1d* marker was placed in front of the *pACT-AMY* expression cassette, two different results were obtained. If the transcription of the genes was divergent (series A), the expression of both genes was severely affected, while if both genes were transcribed in the same direction (series B), their expression was improved. In addition, if the *TRP1d* marker was placed downstream of the *pACT-AMY* cassette (series C), its expression was abolished unless a strong terminator was placed between both genes.

To fulfill the second criteria, integrative transformations should be carried out. However, to obtain high-level expression of the corresponding gene, normally a high copy number of the expression cassette is required. For this reason, multicopy integration of the transformation module should be desirable. The multicopy integration may be obtained if a defective selection marker is used (11–13). In our case we have used a *TRP1d* defective marker since the industrial baker's yeast used as a host was auxotroph to tryptophan. The length of the promoter initially used was changed to obtain a defective marker that was only functional when present in multiple copies. To allow the presence of multiple copies of the transformation module, we targeted its integration to the rDNA locus by placing two ribosomal sequences in the flanks of the fragment. These ribosomal sequences were completely homologous to the ones found in the yeast genomic rDNA locus (see the Materials and Methods).

In this way, we constructed different “integrative cassettes” that were tested for multi-integration. We obtained one transformant called Rib-AMY (CECT10872) that stably maintained a high copy number of the transformation module. This transformant contained around 50–100 copies of the integrative cassette per cell that were located at the rDNA locus. Since only the *pACT-AMY* cassette, the *TRP1d* marker, and the ribosomal sequences were present in the “integrative cassette”, no prokaryotic sequences were found in this transformant, making it suitable for its commercial application. In addition, the Rib-AMY transformant was able to express and secrete enough α -amylase so as to improve bread dough by increasing bread volume and retarding bread firming. This strain can be very useful in bread making, since exogenous supplementation of flour with α -amylase could be avoided, reducing in this way the incidence of allergenic-related symptoms due to the exogenous addition of this enzyme.

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