

High Level Production of Thermostable α -Amylase from *Sulfolobus solfataricus* in High-Cell Density Culture of the Food Yeast *Candida utilis*

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Abstract

The α -amylase from *Sulfolobus solfataricus* has the commercially important ability to hydrolyze glycosyltrehalose and can be used for the production of trehalose from soluble starch. We have produced this enzyme in the food yeast *Candida utilis* at extremely high levels. Because the *S. solfataricus* gene was previously shown to be very poorly expressed, the gene was resynthesized based on codons preferentially found in the highly expressed *C. utilis* glyceraldehyde-3-phosphate dehydrogenase (*GAP*) gene. Expression of this synthetic gene under the control of the *GAP* promoter yielded biologically active α -amylase, accounting for more than 50% of the soluble protein. Comparison of the expression levels of various chimeric constructs of the synthetic and native genes indicated that the production level of the α -amylase was improved more than 2×10^4 -fold by substituting the native gene with the synthesized one. Northern analysis revealed the formation of short mRNAs in transformants with constructs containing native gene fragments, suggesting that premature termination of the transcripts is responsible for the low production level. The α -amylase-producing *C. utilis* cells were grown up to 92 grams dry cell weight per liter in a synthetic medium, yielding 12.3 g/l α -amylase which accounts for up to 27% of total cell proteins.

Sulfolobus solfataricus α -amylase is a glycosyltrehalose-hydrolyzing enzyme which can be used for the production of trehalose, a nonreducing disaccharide with two glucose molecules bound by an α -1,1 linkage (Kato *et al.*, 1996a; Kato *et al.*, 1996b). Trehalose is widely distributed among microorganisms, plants, and insects and has many biological functions such as storage of energy and protection of the cell against heat and osmotic change. Trehalose is used industrially as a preservative material in unstable foods, cosmetics and medicines. Recently, it was found that two enzymes from the hyperthermophilic and

acidophilic archaeum *Sulfolobus solfataricus* KM1, a glycosyltrehalose-producing enzyme (glycosyltransferase) and a glycosyltrehalose-hydrolyzing enzyme (α -amylase), could convert soluble starch to trehalose (Kato *et al.*, 1996a; Kato *et al.*, 1996b). The two genes encoding these enzymes have since been cloned and their nucleotide sequences determined (Kobayashi *et al.*, 1996). Since the *S. solfataricus* enzymes are thermostable, the enzymes produced in the recombinant hosts could be easily separated from the host proteins by simply heating the cell extracts. We therefore attempted to produce one of the enzymes, α -amylase, in the yeast *Candida utilis* in this study.

The yeast *C. utilis* has been used for the production of a number of biologically useful materials, such as amino acids, RNA, glutathione, NAD, and coenzyme A (Boze *et al.*, 1992). It has long been recognized as a useful food resource through the production of SCP (single cell protein) and is approved as a food additive by the FDA. *C. utilis* can assimilate cheap biomass-derived sugars, such as sugar molasses and spent sulfite liquor, and a broad spectrum of compounds as nitrogen sources. Since *C. utilis* does not produce ethanol in strict aerobic culture, large-scale production of cells can be carried out efficiently under continuous culture conditions (Ichii *et al.*, 1993). We have recently developed an integrative transformation system for *C. utilis* using the cycloheximide-resistance (*CYH*^r) gene as a marker, which was constructed from the endogenous ribosomal protein gene L41 by *in vitro* mutagenesis (Kondo *et al.*, 1995a; Kondo *et al.*, 1995b). The vector system makes use of the strong promoter of the glyceraldehyde-3-phosphate dehydrogenase (*GAP*) gene from *C. utilis* and a promoter-deficient *CYH*^r gene that allows high-copy number integration of the vector (Kondo *et al.*, 1997). The major attraction in choosing *C. utilis* as a system for heterologous gene expression is the potential of this organism to produce large amounts of proteins, as has already been demonstrated by our previous study producing the sweet plant protein monellin at high level (Kondo *et al.*, 1997). Recently, we have also described the potential of this yeast as a new producer of astaxanthin, an industrially important carotenoid for pigmenting cultured fish and shellfish (Miura *et al.*, 1998a; Miura *et al.*, 1998b; Shimada *et al.*, 1998). In this study, we describe expression of the α -amylase from *S. solfataricus* in *C. utilis* and its production in high-cell-density fermentation.

Construction of Plasmids and Expression of α -Amylase in *C. utilis*

Early attempts to express the native α -amylase gene from *S. solfataricus* in *C. utilis* resulted in very low levels of expression. Northern analysis indicated that the full-size transcript was hardly detectable despite using a strong promoter from *C. utilis* (data not shown). The A+T content

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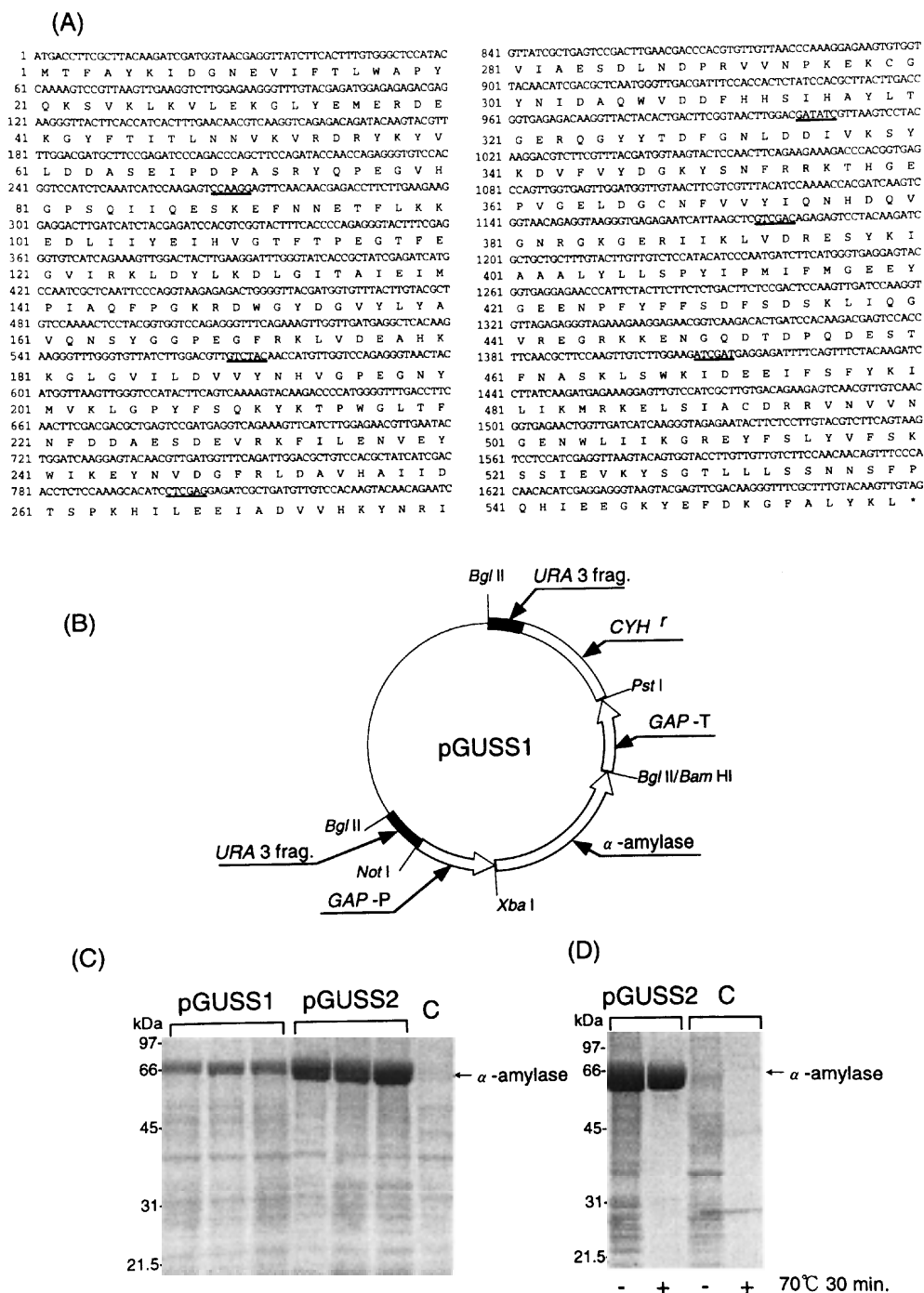


Figure 1. Expression of the α -Amylase in the Yeast *C. utilis*

(A) DNA and deduced amino acid sequences of the synthesized α -amylase gene. The 1587-bp gene was divided into seven fragments of 184 bp to 312 bp and constructed by sequential polymerase chain reaction (PCR) using a total of four oligonucleotides 65 to 100 bases long, which were complementary and partially overlapping. Unique restriction sites were created at the junctions of each fragment for assembly (shown by underline). *Xba*I and *Bgl*II sites were added at the 5' and 3' ends of the open reading frame, respectively. The *Xba*I-*Bgl*II fragment carrying the gene capable of encoding a complete α -amylase was finally assembled by ligating the respective fragments into pUCBgl (Kondo *et al.*, 1997) to construct the plasmid pSS. (B) Structure of the expression plasmid pGUSS1. The *Xba*I-*Bgl*II fragment of pSS was ligated to the *Xba*I and *Bam*HI sites of the pGAPURA1 to construct the pGUSS1. The pGAPURA1 was constructed by inserting a *Not*I-*Pst*I fragment containing the promoter and terminator sequences of the *GAP* gene isolated from the plasmid pGAPPT10 (Kondo *et al.*, 1997) into the *Not*I and *Pst*I sites of pURAL10, which contains the *CYH*^r gene as well as the 5' and 3' fragments of the *URA3* gene from *C. utilis* (Kondo *et al.*, 1997). The plasmid pGUSS2 for high level production of α -amylase was constructed in the same manner using the pURAL11 (Kondo *et al.*, 1997). The plasmid was used for transformation after cutting at the *Bgl*II sites to stimulate integration. (C) Expression of α -amylase. Cells cultured in YPD medium were resuspended in 50mM sodium-acetate (pH 5.5) containing 0.3 mg/ml Zymolyase 100T (Seikagaku Kogyo Co., Ltd.) and incubated at 37 °C for 30 min. Cells in suspension were disrupted by vortexing for 5 min with glass beads (diameter, 400-600 μ m; Sigma Chemical Co., Ltd.). Cell debris was removed by centrifugation at 12,000g for 10 min. The soluble fractions were subjected to SDS-PAGE and stained with Coomassie brilliant blue. The plasmids used for transformation are indicated above the gel. C is a control strain transformed with pCLRE2 (Kondo *et al.*, 1995b). (D) Purification of the α -amylase by heat treatment. The extracts from the transformants with pGUSS2 and pCLRE2 (Kondo *et al.*, 1995b) were treated at 70 °C for 30 min and centrifuged at 12,000g for 10 min to remove the precipitated proteins. The supernatants were subjected to SDS-PAGE and stained with Coomassie brilliant blue.

of the *S. solfataricus* α -amylase gene is significantly higher (63%) (Kobayashi *et al.*, 1996) than that of 5 cloned genes of *C. utilis* (52%) (unpublished data). The 1587-bp gene encoding the 528-amino acid α -amylase gene was therefore synthesized without changing its original amino acid sequence based on codons preferentially found in the *GAP* gene (Kondo *et al.*, 1995a; Kondo *et al.*, 1997), which is a highly expressed gene in *C. utilis* (Figure 1A).

The synthesized α -amylase gene was connected to the promoter and terminator sequences of the *GAP* gene to construct the expression cassette in the plasmid pGUSS1 as shown in Figure 1B. The plasmid also contains the *CYH^r* gene as a selection marker, constructed by a point mutation in the *C. utilis* L41 gene (Kondo *et al.*, 1995b), and *URA3* gene fragments as target sequences for plasmid integration into the chromosome. Plasmid pGUSS2 was constructed to further increase the expression level of the gene. This plasmid has essentially the same structure as pGUSS1 except for the *CYH^r* gene, which has a defective promoter due to a deletion from its 5' end. Transformation of the cells with this plasmid previously resulted in a high integration number (Kondo *et al.*, 1997). Both plasmids have structures in which digestion with *Bgl*II generates linear DNA with recombination ends (*URA3*) that facilitate chromosome integration. Thus the plasmids integrate at the *URA3* locus without the accompanying bacterial sequences.

C. utilis ATCC 9950 strain was transformed with the plasmids as previously described (Kondo *et al.*, 1995b). Three independent *CYH*-resistant clones transformed with either plasmid pGUSS1 or pGUSS2 were cultured in 10 ml of YPD medium (1% yeast extract, 2% peptone and 2% glucose) containing cycloheximide (40 μ g/ml). Cell extracts were prepared and soluble proteins were subjected to SDS-PAGE (Figure 1C). With both plasmids, an intense band at 55kDa corresponding to the expected molecular weight of α -amylase was detected. The production level of α -amylase in transformants with the high-copy enhancing pGUSS2 was much higher than with pGUSS1. Quantitative scanning of the gel indicated that the α -amylase accounts for more than 50% of the total soluble protein in the transformants with pGUSS2. The production level of α -amylase was not less than that of monellin (Kondo *et al.*, 1997). Since the *S. solfataricus* α -amylase is a thermostable enzyme, it was easily separated from the host proteins by heat treatment, as determined by analyzing the soluble proteins by SDS-PAGE after treating the cell extracts at 70 °C for 30 minutes (Figure 1D). Southern analysis using the L41 gene fragment as a probe indicated that the copy number of the integrated DNA fragments was 10 to 11 copies for pGUSS1, and 15 to 22 copies for pGUSS2. The absence of bacterial sequences in the transformants was confirmed by Southern analysis (data not shown). Stability of the integrated DNA fragments in four independent clones transformed with pGUSS2 was examined by repeating the culture in non-selective medium. No detectable decrease in the α -amylase production level was observed after 50 generations of non-selective growth (data not shown), consistent with the stability previously demonstrated for the monellin-producing strain (Kondo *et al.*, 1997). The ratio of cells retaining resistance to cycloheximide to the total number of cells after 50 generations of non-selective growth was between 80% and 100% (data not shown).

Production of α -Amylase with High Cell-Density Cultivation

The α -amylase-producing cells transformed with pGUSS2 were grown in a 2.5-liter jar fermenter with successive feeding of concentrated nutrients. Samples were taken at periodic intervals and the dry cell weights and enzyme activities were measured (Figure 2A). Cells were grown up to 92 g dry weight per liter of culture medium and the amount of α -amylase reached up to 12.3 g per liter (27% of total cell proteins) after 59h of culture. The amounts of α -amylase produced in the cells were estimated by comparing the measured activities with the specific activity of the purified enzyme. Throughout the cultivation, soluble proteins analyzed by SDS-PAGE showed an intense band at 55kDa corresponding to the α -amylase (Figure 2B), with a little degradation observed at the end of the run (59h).

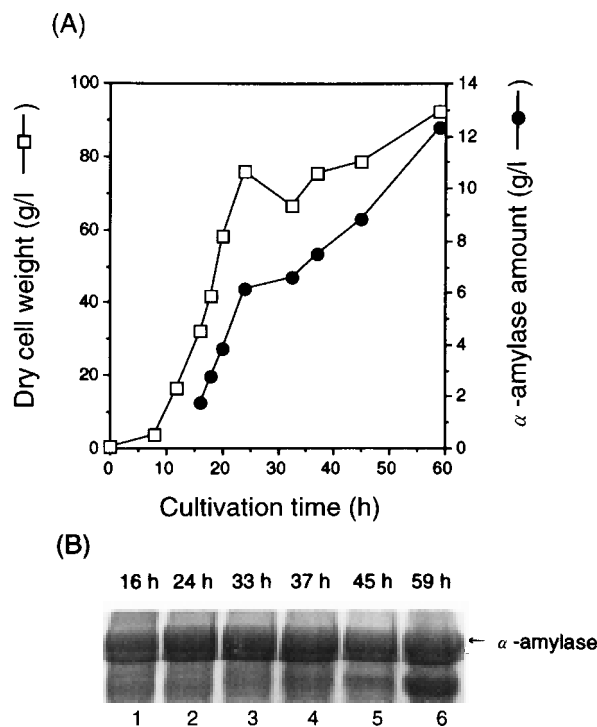


Figure 2. High-Cell-Density Cultivation of the *C. utilis* Strain with pGUSS2 (A) Production of α -amylase in a 2.5 liter fed-batch fermentation showing the cell densities (dry weight gram/liter) and the amount of α -amylase in the cells (gram/liter) as a function of time. Seed cultures, grown in 100 ml of YPD medium in a 500-ml conical flask for 72 hr at 30 °C, were used to inoculate 1.5 l of a modified SD medium (3% glucose, 0.5% $(\text{NH}_4)_2\text{SO}_4$, vitamin, and rare metals) in a 2.5 liter jar fermenter (Tokyo Rika Co., Ltd.). The fermenter was operated at 30 °C and the pH maintained at 5.0 by adding NH_4OH . A concentrated medium containing glucose, $(\text{NH}_4)_2\text{SO}_4$, rare metals, and vitamins was successively fed to the growing cells to obtain a high-cell-density culture. The glucose concentration was kept between 1% and 5%. Samples were taken at each time point to measure the dry weight of cells and enzyme activities. The dry weight of cells was measured after lyophilization. 0.3 g of wet cells was resuspended in 1 ml of 10mM sodium phosphate (pH 7.5) containing 0.15 mg of Zymolyase 100T and shaken at 40 °C for 3 hr and then at 70 °C for 1 hr. The solution was centrifuged at 12,000g for 10 min to remove cell debris. Enzyme activity of the supernatant was measured according to the method described by Kato *et al.*, (1996a). The weight of α -amylase was calculated according to the specific activity of the purified α -amylase of 484.6 units/mg (unpublished data). (B) The protein extracts prepared from the cells collected at the indicated times were subjected to SDS-PAGE and stained with Coomassie brilliant blue.

The amounts of α -amylase estimated from the Coomassie-stained gel and from the calculation of enzyme activity were in agreement, indicating that the enzyme produced in *C. utilis* is biologically active.

The production level of α -amylase is the highest of which we are aware for the production of an active recombinant protein using yeast expression systems. There has been only one report that showed comparable production level of a recombinant protein in a yeast expression system, the production of the tetanus toxin fragment C produced in *Pichia pastoris* which reached 12 g/l at cell densities of 90 g/l (Clare *et al.*, 1991; Romanos *et al.*, 1992). Further study of the fermentation conditions of the α -amylase-producing strain could possibly enable even higher production levels, since high cell density cultivation at 120 g/l dry cells has been attained using a *C. utilis* strain (unpublished data). The enzyme's high concentration and its stability at high temperature resulted in easy purification from the host proteins, significantly reducing the production cost. Furthermore, the efficient production of recombinant proteins and ease of high density culture make the yeast *C. utilis* an excellent host for commercial production of enzymes.

Effect of Gene Synthesis on α -Amylase Gene Expression

Various chimeras of the native and synthetic genes were constructed in order to investigate the effect of gene synthesis on expression. The plasmids pGUNS1, pGUSN1, and pGUNN1, in which the α -amylase gene fragment in the pGUSS1 is substituted with the various chimeric genes, were constructed and used for transformation of *C. utilis* (Figure 3). The sequence of the 54 bp at the 5' region of each gene is identical, allowing each transcript to be detected with the same probe. Two clones, in which approximately ten copies of the DNA fragments were integrated, were selected among the transformants obtained with each plasmid. The copy-number of each integrated DNA fragment was estimated by Southern analysis using the L41 gene as a probe (data not shown). The clones were cultured in 10ml of YPD medium and the

α -amylase was purified from the cell extracts by centrifugation after a 30 min treatment at 70 °C. A band corresponding to α -amylase on SDS-PAGE was hardly detectable in the extracts of the transformants of pGUNN1 containing the native *S. solfolobus* gene (Figure 4A, lanes 7 and 8). Faint bands of α -amylase were visible in the extracts with pGUNS1, in which the 3' two-thirds (1045 bp) of the native gene was replaced with the synthetic gene (lanes 3 and 4). Replacement of the 5' two-thirds (1058 bp) of the native gene with the synthetic fragment significantly improved expression and the bands are distinctly observed (pGUSN1, lanes 5 and 6), although the production level was still much lower than that of pGUSS1 (lanes 1 and 2). Measurement of α -amylase activity revealed that replacement of either side of the native gene with the synthetic one in pGUNS1 and pGUSN1 resulted in 217-fold and 982-fold increase in activity, respectively (Figure 4A). However the levels were considerably lower (0.8 % and 3.9 %, respectively) than that of pGUSS1 which carries the complete synthetic gene. The production level of α -amylase in pGUSS1 was 2.5×10^4 -fold greater than that of pGUNN1. It should be noted that the relative enzyme activities were in good agreement with the relative intensities of the protein bands. These results show that a gene with high A+T content must be completely synthesized based on the codon usage of *C. utilis* to attain high levels of expression in *C. utilis*.

To investigate whether the differences in enzyme production among the constructs could be explained by variations in steady-state mRNA levels, Northern blot analysis was carried out. A DNA fragment of 54 bp, which corresponds to the 5' part of the synthetic α -amylase gene and is conserved in each construct (Figure 3), was used as a probe. An ethidium bromide-stained gel confirmed that the relative amount of RNAs loaded in each lane was almost equal (Figure 4B, bottom panel). After a short exposure, a mRNA band of 1.7 kb, corresponding to the size of the full-length α -amylase gene, was detected only in cells containing pGUSS1 and pGUSN1 (upper panel, lanes 1,2 and 5,6). An additional truncated mRNA band of 1.0 kb was also detected in RNA derived from pGUSN1 (upper panel, lanes 5 and 6). With pGUNS1 and pGUNN1,

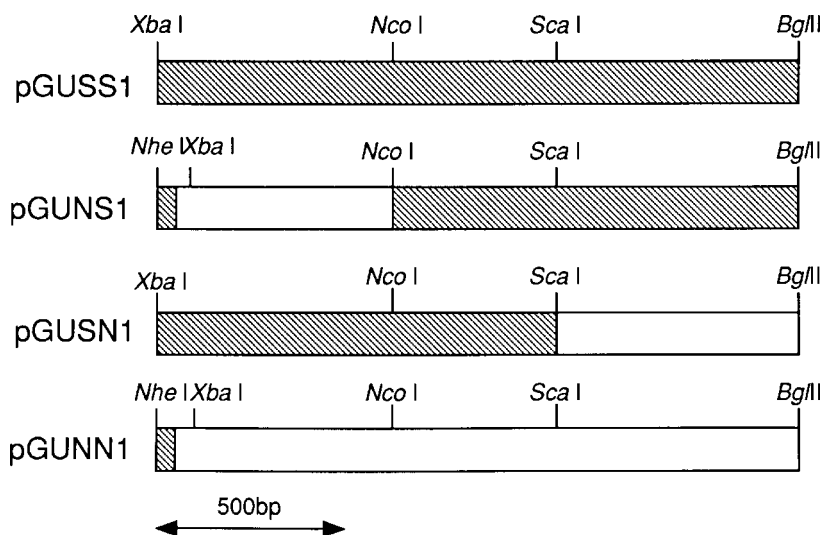


Figure 3. Structures of the α -Amylase Genes in the Expression Plasmids

The α -amylase gene fragments in the expression plasmids are shown. The open and hatched boxes denote the native and synthetic sequences, respectively. The plasmids pGUSS1, pGUNS1, pGUSN1, and pGUNN1 were constructed by ligating the *NheI*-*BglII* or *XbaI*-*BglII* fragments of the α -amylase gene from the plasmids pSS, pNS, pSN, and pNN into the *XbaI* and *BamHI* sites of pGAPURA1, respectively. PCR was performed on a plasmid containing the native *S. solfataricus* α -amylase gene (Kobayashi *et al.*, 1996) to add *NheI* and *BglII* sites at the 5' and 3' ends of the ORF and also to substitute 54 bp at the 5' end of the native gene with the synthesized sequence from pSS. The amplified fragment was inserted into the *HindIII* site of pUC118 to construct plasmid pNN. Plasmids pNS and pSN were constructed from pSS and pNN by combining DNA fragments obtained from both plasmids.

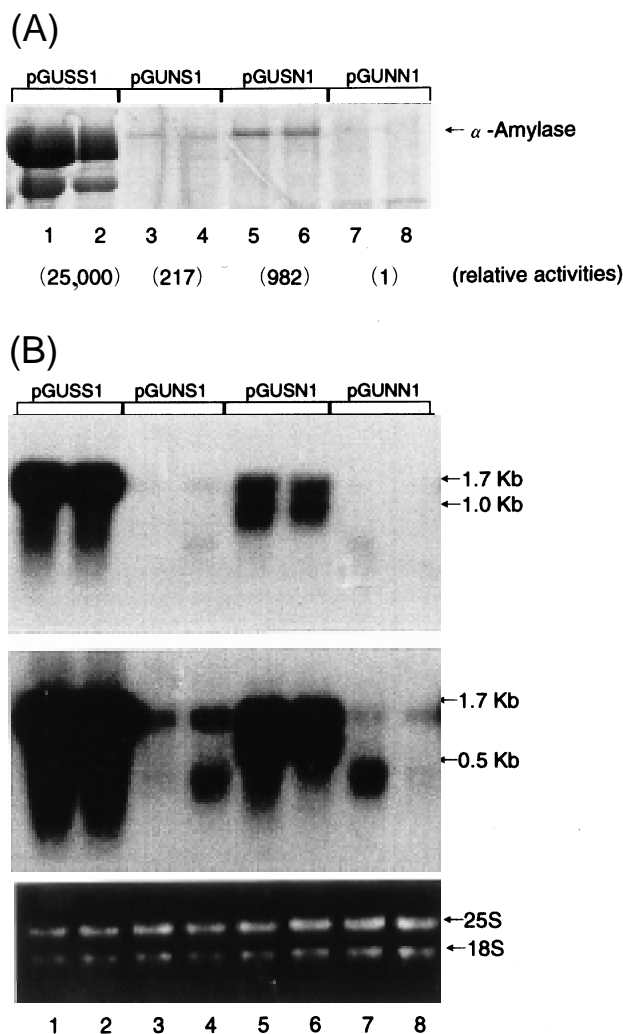


Figure 4. Expression Analysis of α -Amylase with the Different Gene Constructs

(A) Analysis of proteins in the cell extracts of the transformants. For each plasmid, two transformants were cultured in YPD medium and the cell extracts were prepared. The extracts were further treated at 70 °C for 30 min and centrifuged to remove the insoluble proteins. The supernatants were subjected to SDS-PAGE and stained with Coomassie brilliant blue. The area including the α -amylase bands is shown. The plasmids used for transformation are indicated at the top of the gel and the relative enzyme activities are shown on the bottom. (B) Northern hybridization analysis of the RNAs of the transformants. Total RNA was isolated from cells by the guanidine-thiocyanated method (Sambrook *et al.*, 1989). Ten μ g of the RNA was electrophoresed on a 1.2% agarose/formaldehyde gel and the ethidium-bromide stained gel is shown on the bottom. RNAs transferred to a Hybond-N+ membrane (Amersham) were hybridized with the probe DNA, as described previously (Sambrook *et al.*, 1989). The 54-bp fragment corresponding to the 5' end of the synthetic α -amylase gene was 32 P-labeled using MegaPrime DNA Labelling System (Amersham) as described by the supplier. The filters were subjected to autoradiography on X-Ray film. The top panel shows the result after a short exposure to film and an overexposed film is shown in the middle panel. RNA markers were used for estimation of the band sizes.

no bands were detected after a short exposure (upper panel, lanes 3,4 and 7,8), and the full-length mRNA was barely observed along with a shorter transcript of 0.5 kb after a longer exposure (middle panel, lanes 3,4 and 7,8). The presence of the truncated mRNAs of 1.0-kb observed in pGUSN1 and of 0.5-kb in pGUNS1 and pGUNN1 is most likely due to premature termination of transcription in the

A+T rich native sequences. Only the plasmid pGUSS1 containing the synthetic gene gave large amounts of the full-length mRNA as the major species (Figure 4B, lanes 1 and 2).

Similar results have been reported for the expression of A+T rich genes in *Saccharomyces cerevisiae* and *Pichia pastoris*, where protein expression could not be observed and truncated mRNAs were detected by Northern analysis of the RNAs (Romanos *et al.*, 1991; Scorer *et al.*, 1993). The A+T rich sequences in the native genes seemed to function as premature polyadenylation sites, resulting in the production of truncated RNA species (Romanos *et al.*, 1991; Scorer *et al.*, 1993). In contrast to previous reports showing the accumulation of essentially the same amounts of full-length and truncated mRNAs, our results show a much larger proportion of full-length mRNA than truncated mRNAs. The considerable decrease in mRNA in the chimeric gene constructs, pGUSN1, pGUNS1 and pGUNN1, suggests that the mRNAs with native gene sequences are unstable in the cell, preventing the accumulation of the truncated transcripts.

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