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Expression of *Pseudomonas amyloderamosa* isoamylase gene in *Saccharomyces cerevisiae*

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Isoamylase gene (*iso*) of *Pseudomonas amyloderamosa* was amplified by polymerase chain reaction and cloned into *Saccharomyces cerevisiae* vectors under the control of alcohol dehydrogenase gene and glyceraldehyde-3-phosphate dehydrogenase gene promoters. The signal sequence of *iso* gene was also replaced with that of *Schwanniomyces occidentalis* α-amylase gene. The extracellular isoamylase activity of transformed *Sacc. cerevisiae* could reach 86 U ml^{-1} after a 4-days cultivation.

Introduction

Isoamylase (E.C. 3.2.1.68) is a debranching enzyme that hydrolyzes both inner and outer branching linkages of amylopectin (Harada *et al.*, 1972) and is capable of improving the saccharification of starch to produce commercial important products (Harada, 1984). *P. amyloderamosa* is known to produce industrially useful isoamylase (Harada, 1984). The enzyme possesses the ability to bind specifically to raw starch and the adsorbed isoamylase can be eluted by acetate buffer containing 10% maltose (Fang *et al.*, 1994). We have reported the molecular cloning and DNA sequence of the isoamylase gene from *P. amyloderamosa* (Chen *et al.*, 1990). The cloned gene was expressed in *Escherichia coli* (Lin and Chu, 1995). The present report describes the construction of recombinant plasmids containing *iso* gene from *P. amyloderamosa* and the functional expression of isoamylase in *Sacc. cerevisiae*.

Materials and methods

Microorganisms, vectors and growth conditions

E. coli strain HB101 (*F*$^-$ *rec*A *pro leu*B *thi lac*Y *str* r_k^- m_k^-) (Sambrook *et al.*, 1989) was used as a host in plasmid construction. *P. amyloderamosa* SB15, *Sacc. cerevisiae* AH22 and *Schw. occidentalis* were obtained from Culture Collection and Research Center (Hsinchu, Taiwan). Plasmids used were pBluescript II KS+ (Stratagene Co., CA, USA), pAAH5 (Aminerer, 1983) and pG3 (Yamamoto *et al.*, 1989). *E. coli* was grown in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989). *E. coli* harboring *iso* gene was screened for blue zone formation around the colony on a complex medium (2% amylopectin, 0.2% peptone, 0.3% $(NH_4)_2HPO_4$, 0.05% $MgSO_4 \cdot 7H_2O$ and 1.5% agar; pH 5.0) after treated with iodine vapor. The growth condition for *P. amyloderamosa* SB15 was described previously (Chen *et al.*, 1990). For isoamylase production, yeast cells were grown in YPD medium (1% yeast extract, 2% peptone and 2% glucose) and shaken at 30°C.

DNA manipulations and transformation procedures

The isolation of *P. amyloderamosa* chromosomal DNA was essentially performed by the method of Amemura *et al.* (1988). The procedure used for preparation of *Schw. occidentalis* chromosomal DNA has been described elsewhere (Sherman *et al.*, 1986). The digestion of DNA with restriction enzymes and other routine molecular methods used in this work were described by Sambrook *et al.* (1989). *Sacc. cerevisiae* AH22 was transformed with recombinant plasmid DNA by the lithium acetate method (Gietz *et al.*, 1992).

Construction of expression plasmids

The *iso* gene was amplified from *P. amyloderamosa* SB15 chromosomal DNA by PCR using primers ISO-4 (5'-GGAAAGCTTCTGACCCATGAAGTGCCCAAA-3') and ISO-5 (5'-GGGAAGCTTACAAACACGTCAGCCAGACCT-3'). PCR amplification of *iso* gene was performed in 100 μl of a solution containing 10 mM Tris/HCl (pH 8.3), 1.25 mM $MgCl_2$, 200 mM each dNTP, 5 pmol each primer, 50 ng chromosomal DNA and 2.5 units *Taq* DNA polymerase (Amersham, Buckinghamshire, UK). The reaction mixture was incubated in a GeneAmp model 9600 thermocycler (Perkin-Elmer Cetus, Norwalk, USA) for 30 cycles of 1.5 min of denaturation at 94°C, 2 min of annealing at 65°C and 2 min of elongation at 74°C and a

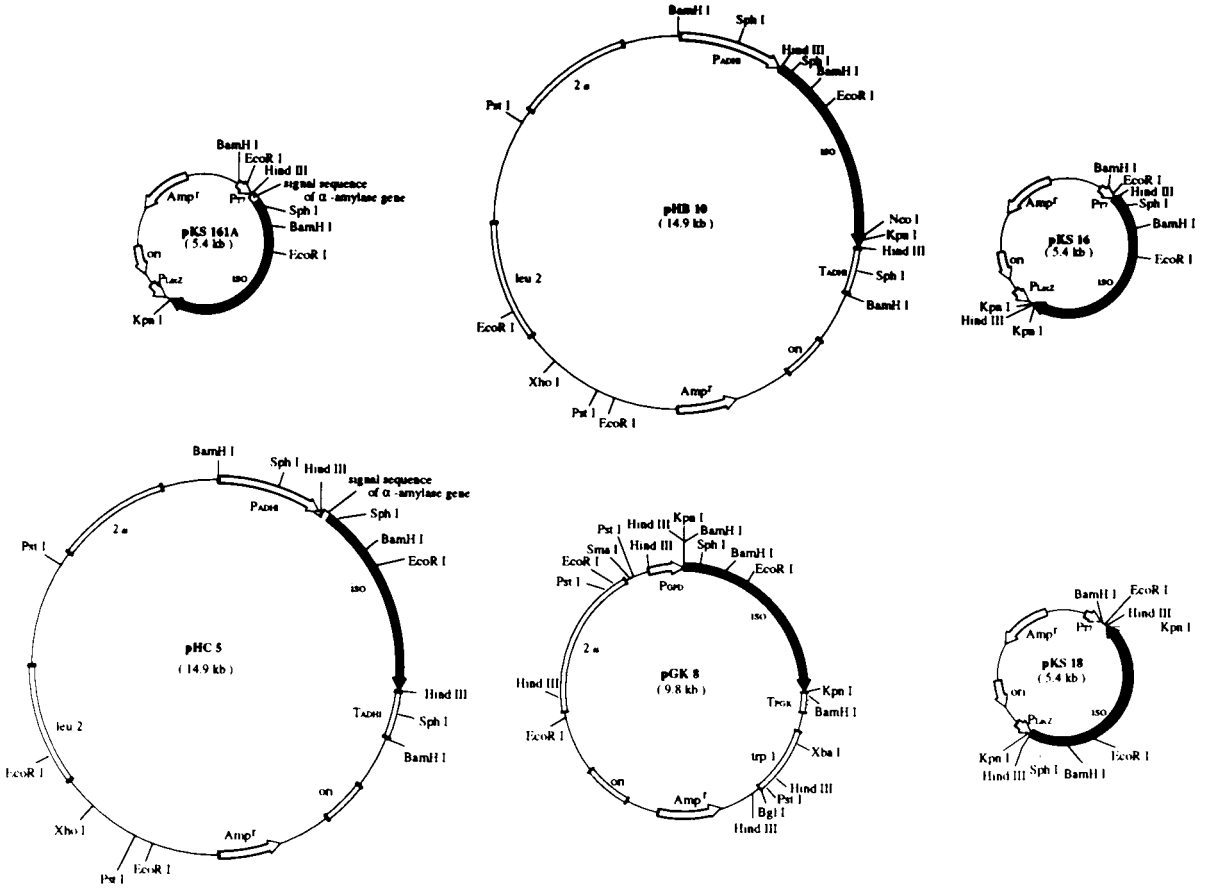

Figure 1 Plasmids used to express *iso* gene in *Sacc. cerevisiae*. Symbols denote: P_{T7}, T7 promoter; P_{lacZ}, *lac*Z promoter; P_{adh1}, alcohol dehydrogenase promoter; P_{gpd}, glyceraldehyde-3-phosphate dehydrogenase promoter; Ori, bacterial origin of replication; Amp, ampicillin-resistant marker; Iso, isoamylase gene; 2μ, autonomous replicating sequence form yeast 2μ plasmid. The transcriptional direction of the *iso* gene is indicated by an arrow.

final elongation for 10 min at 74°C. The PCR product was recovered by the Geneclean II kit (Bio 101, Inc., CA, USA), digested with *Hin*dIII and cloned into pBluescript II KS+ to construct plasmids pKS16 and pKS18. The *iso* gene was then released from pKS16 by *Hin*dIII and cloned into the *Hin*dIII site of pAAH5 to yield pHB10 (Fig. 1). A 2.4-kb DNA fragment containing the complete coding sequence of *iso* gene was prepared by digesting pKS18 with *Kpn*I. Plasmid pGK8 was then constructed by ligating this fragment into pG3. To replace the signal sequence of isoamylase, a 100-bp DNA fragment encoding the first 30 amino acids of *Schw. occidentalis* α-amylase gene was amplified by PCR with primers AMYS-1 (5'-GGAAGCTTAGATCTATGAGATTTCAACTGAAGGAT-3') and AMYS-2 (5'-TAATCGGTTGAGCATGCGCCA-ATCTTGAGA-3'). After digestion with *Hin*dIII and *Sph*I, this fragment was then subcloned into the corresponding sites of pKS16, in which the *Hin*dIII site between two *Kpn*I sites had been deleted by *Kpn*I digestion, to construct pKS161A. The recombinant *iso* gene was amplified from pKS161A by PCR and subcloned as a 2.4-kb *Hin*dIII DNA fragment into the *Hin*dIII site of plasmid pAAH5. The resulting plasmid pHC5 containing the signal sequence of *Schw. occidentalis* α-amylase gene was verified by DNA sequencing.

Enzyme assay and localization of isoamylase

Isoamylase activity was determined by the method described by Houng *et al.* (1989) with some modifications. The reaction mixture consisted of 350 μl 0.5% amylopectin, 100 μl 0.5 M sodium acetate (pH 3.5) and 50 μl enzyme solution. The reaction was performed at 40°C for 10 min and stopped by the addition of 500 μl iodine solution (0.01 M I_2 – 0.1 M KI). After a 10-fold dilution with distilled water, the absorbance of the reaction mixture was measured at 610 nm. One unit of isoamylase activity was defined as the amount of the enzyme giving an increase in absorbance of 0.01 per min. To investigate the distribu-

tion of isoamylase activity, *Sacc. cerevisiae* harboring pHB10, pGK8 or pHC5 was inoculated in 100 ml YPD medium and grown at 30°C for 24, 48, 72 and 96 h, respectively. The cells were harvested by centrifugation at 9,000 × *g* for 10 min and supernatant fluids were assayed for isoamylase activity. Pellets were washed once with 50 mM acetate buffer (pH 3.5), resuspended in 2 ml of the above buffer and disrupted by the method of Nam *et al.* (1993). As a control for *Sacc. cerevisiae* intracellular enzyme, glyceraldehyde-3-phosphate dehydrogenase was assayed by the method of Ferdinand (1964).

Results

The *iso* gene of *P. amyloderamosa* SB15 was PCR-amplified and cloned as a 2.4-kb fragment into plasmid pBluescript KS+ to generate plasmids pKS16 and pKS18 (Fig. 1). Competent *E. coli* HB101 cells were transformed with the recombinant plasmids to ampicillin resistance and screened for amylopectin hydrolysis. As shown in Fig. 2, *E. coli* HB101 harboring pKS16 exhibited a blue zone when the amylopectin plate was visualized by iodine vapor, indicating that the *iso* gene of *P. amyloderomosa* was functionally expressed in *E. coli*.

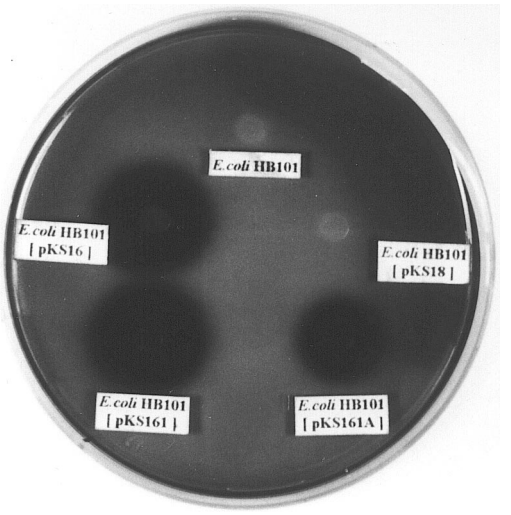

Figure 2 Secretion of isoamylase by *E. coli* transformants. Transformants were spotted on a complex medium containing 2% (w/v) amylopectin and cultivated at 37 °C for 2 days. The colonies showing a blue zone by iodine staining indicate the expression of the *iso* gene.

The *iso* gene including its own signal sequence was subcloned from pKS16 and pKS18 into the pAHH5 or pG3 vector. The recombinant plasmids, pHB10 and pGK8, directed the transcription of *iso* gene under the control of *adh*1 and *gpd* promoters, respectively. To determine the effect of the nature of the signal peptide on the efficiency of enzyme secretion, we also constructed an expression plasmid pHC5 in which the signal sequence of isoamylase was replaced by a signal sequence from *Schw. occidentalis* α-amylase. *Sacc. cerevisiae* was transformed with pHB10, pGK8 or pHC5 and Leu^+ transformants were selected. Transformants were further examined for isoamylase synthesis on YPD agar containing 2% (w/v) amylopectin by iodine staining (data not shown). The colonies showing a blue zone were isolated and then used for isoamylase production.

Sacc. cerevisiae containing *iso* fused to either *adh*1 or *gpd* promoters exhibited isoamylase activity on amylopectin-PAGE while no extracellular enzyme activity was obtained by the parent strain (data not shown). To quantify the isoamylase production by various transformants, a time course experiment was performed using liquid medium. The individual transformants gave a similar cell concentration at the end of cultivation (Table 1), indicating independency of cell growth on the plasmid or promoter type. It was observed that low level of isoamylase was secreted during the first 24 h. Of the transformants, the isoamylase

Table 1 Distribution of isoamylase activity in *Sacc. cerevisiae* transformants.

Plasmid	Cell mass[a] (mg l^{-1})	Fraction	Enzyme activity (U ml^{-1}) 24 h	48 h	72 h	96 h
pGK8	1.51	Supernatant	3.0 (33%)[b]	19.3 (49%)	37.6 (54%)	86.5 (58%)
		Intracellular	6.1 (67%)	20.4 (51%)	33.2 (46%)	62.4 (42%)
pHB10	1.59	Supernatant	0.3 (27%)	4.2 (45%)	19.1 (47%)	52.3 (53%)
		Intracellular	0.8 (73%)	5.1 (55%)	21.2 (53%)	47.2 (47%)
pHC5	1.62	Supernatant	2.0 (53%)	5.3 (60%)	31.9 (69%)	84.6 (74%)
		Intracellular	1.8 (47%)	3.5 (40%)	15.8 (31%)	29.7 (26%)

[a] Cell mass was determined after cultivation at 30°C for 96 h.
[b] Numbers in parentheses represent the percentage of total enzymatic activity in the respective fractions.

activity of *Sacc. cerevisiae* carrying pGK8 reached a maximum of 86 U ml^{-1} after 4-days cultivation. The recombinant plasmids, pHB10 and pHC5 contained the *P. amyloderamosa* isoamylase signal peptide and *Schw. occidentalis* α-amylase signal sequence, respectively, and directed the transcription of *iso* gene under the control of yeast *adh*1 promoter (Fig. 1). Comparing the isoamylase production of their transformants, it was found that the yeast-derived signal sequence did significantly improve the secretion of isoamylase. After cultivation at 30°C for 96 h, the total isoamylase activity of extracellular fractions reached approximately 74% while less than 47% of enzymatic activity was detected intracellularly (Table 1). It is worth to note that no significant glucose-induced glyceraldehyde-3-phosphate dehydrogenase activity was detected in the supernatant fraction at the end of fermentation. This observation indicated that cell lysis could not be happened in *Sacc. cerevisiae* harboring *iso* gene.

Discussion

We have demonstrated the expression of *P. amyloderamosa iso* gene in *Sacc. cerevisiae* using the yeast promoters. Although the *Bacillus amyloliquefaciens* α-amylase gene was expressed in yeast without using yeast promoter (Pretorius *et al.*, 1988), we had found that the promoter of the *P. amyloderamosa iso* gene did not function in *Saccharomyces* (data not shown). Previously, the glucoamylase gene from *Aspergillus awamori* was expressed in *Sacc. cerevisiae* only if the promoter and termination regions from a yeast enolase gene was used (Innis *et al.,* 1985), and the expression of *E. coli* xylose isomerase gene required the *trp*5 yeast promoter (Ho *et al.*, 1983). We have used, respectively, the *adh*1 and *gpd* yeast promoters for the expression of *P. amyloderamosa iso* gene. *Sacc. cerevisiae* harbouring either pHB10 or pGK8 had the extracellular isoamylase activity of 52 and 86 U ml^{-1}, respectively. A higher production of isoamylase by *Sacc. cerevisiae* (pGK8) could be due to a stronger transcription ability possessed by the *gpd* promoter (Bennetzen and Hall, 1982).

There are some instances of bacterial polysaccharide-degrading enymes that have been secreted by *Sacc. cerevisiae* using yeast signal peptide derived from glucoamylase (Uozumi *et al.*, 1993), α-galactosidase (Wong *et al.*, 1988) and *Mfα1* gene (Nonato and Shishido, 1988). A limited secretion of *Klebsiella pneumoniae* pullulanase in *Sacc. cerevisiae* was observed using the signal sequence of the yeast mating pheromone α-factor (Janse and Pretorious, 1993). In our case, *Sacc. cerevisiae* is able to secreting the *P. amyloderamosa* isoamylase under the direction of its native signal peptide. However, the use of *Schw. occidentalis* α-amylase signal sequence resulted in a higher level production of isoamylase (Table 1). A possible explanation is that the use of an authentic yeast signal peptide could result in an efficient secretion of foreign polypeptides.

Sacc. cerevisiae is widely used in commercial production of alcoholic beverages or ethanol from starch-containing raw materials. However, it lacks the amylolytic enzymes necessary for starch utilization. Studies are in progress to construct an amylolytic yeast by multiple integration of *A. awamori* glucoamylase and *P. amyloderamosa iso* genes into *S. cerevisiae* chromosome. The recombinant strain can be of great potential for an one-step bioconversion of starchy materials to commercial important products.

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