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Improvement of Heterologous Protein Productivity Through a Selected Bioprocess Strategy and Medium Design

A Case Study for Recombinant *Yarrowia lipolytica* Fermentation

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ABSTRACT

The effect of polypeptide fractions of proteose peptone on the induction of cloned gene expression of rice α-amylase in recombinant *Yarrowia lipolytica* which is under the control of its *XPR2* promoter, was studied. Gel-filtration chromatography with Sephacryl S-100 and Sephadex G-25 (coarse) gels was used to fractionate the active polypeptide fractions from the proteose peptone. The polypeptide size fractions that were effective for the induction of cloned gene expression ranged between mol wt of 1.0 and 6.0 kDa. The fed-batch culture experiments with active polypeptide fractions were performed in a 6-L fermenter. The specific productivity of α-amylase and the enzyme yield based on nitrogen source increased from 25.7 to 33.0 U/g cell·h and 4.96 to 6.73 U/(mg nitrogen consumed), respectively, when proteose peptone was replaced by active polypeptide fractions in production medium. The specific productivity of α-amylase and the enzyme yield further improved to 36.2 U/g cell·h and 8.14 U/(mg nitrogen consumed), respectively, when the glutamic acid-enriched active polypeptide fractions in the production

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medium was used. The specific productivity of α-amylase and the enzyme yield were improved by 41 and 64%, respectively, as compared with the results obtained with the medium containing proteose peptone. Through medium design, a bioprocess strategy for heterologous protein production was developed and a significant productivity improvement achieved.

Index Entries: Recombinant *Yarrowia lipolytica;* heterologous protein; rice α-amylase; medium design for recombinant; enhancement of protein production; active polypeptide fractions; bioprocess strategy; fed-batch fermentation.

INTRODUCTION

Regulatable promoter systems provide the ability to "turn on" the expression of a foreign gene by manipulating defined environmental signals, such as temperature *(1–3)*, medium components *(4,5)*, or the concentration of a particular component in the growth medium *(6,7)*. For example, the bacteriophage λ P_L and P_R promoters working with a temperature-sensitive cI_{857} repressor gene can be induced when the temperature of the culture broth is raised from the growth-optimum temperature of 37°C to 42°C *(2,3)*. Similarly, the *lac* promoter is induced in the presence of lactose or isopropyl-β-D-thiogalactoside (IPTG) *(8)*.

The *SUC2* and *PHO5* promoters of *Saccharomyces cerevisiae* are induced following a low glucose or sucrose level and the depletion of free inorganic phosphate (P_i), respectively, from the medium *(6,7)*. The *Pichia pastoris AOX1* promoter is induced by shifting glucose, glycerol, or ethanol to methanol as the sole source of carbon *(4,9)*. In a glycerol and minimal salts medium, the *Yarrowia lipolytica XPR2* promoter is induced by switching ammonium sulfate to proteose peptone as the sole source of nitrogen *(7)*. The ability to induce foreign gene expression at will allows one to separate the cell-growth phase from the production phase and optimally control the fermentation process.

Y. lipolytica is one of several yeast strains currently considered and examined as alternative hosts for foreign gene expression *(10)*. The *XPR2* gene encodes an inducible alkaline extracellular protease (AEP), which is the major protein secreted by this yeast strain *(11)*. Derepression of AEP occurs at pH 6.0–7.5 on medium lacking preferred carbon, nitrogen, or sulfur sources in a shift-down experiment. The *XPR2* promoter is induced in the presence of proteose peptone in the culture medium. In a glycerol and minimal salts medium containing proteose peptone, AEP production is 800 times higher than the medium containing ammonium sulfate as the nitrogen source *(5)*. The exact nature of the inducer and its regulatory

mechanism have not been elucidated, although certain polypeptide components of the proteose peptone appear to be the major factors.

The *XPR2* promoter has been used to express various cloned genes in *Y. lipolytica*, because it is a very strong and well-regulated promoter *(12,13)*. The difficulty with expression of foreign proteins using the *XPR2* promoter is that the host strain must have an inactive copy of the *XPR2* gene in order to prevent degradation of the foreign protein by AEP. Such a mutant strain cannot grow well on media containing high-mol-wt polypeptides that support the highest level of AEP production in wild-type strains. Therefore, media containing low-mol-wt polypeptides, such as proteose peptone and peptone, have been used to induce cloned gene expression in recombinant *Y. lipolytica (12,14)*. Some components of the proteose peptone and peptone may have an inducer effect *(12,15)*. Proteose peptone and peptone contain low-mol-wt peptides and free amino acids. They may repress foreign protein expression in recombinant strain of *Y. lipolytica*, since they can be easily assimilated *(12)*. Therefore, in the presence of low-mol-wt peptides and free amino acids, it appears that the *XPR2* promoter is not fully induced and results in a low yield of foreign proteins. To induce and express heterologous proteins fully, the use of active polypeptide fractions of proteose peptone is considered very important for practical applications.

Recently, *Y. lipolytica* has become an attractive microbial host for the expression and secretion of foreign proteins. Currently, *Y. lipolytica* expression systems, such as integrative vector, replicative plasmid, and multiple-copy integration, are now available, and the level of protein expression can be manipulated by the gene dosage *(12–14,16)*. A great deal of effort has focused on maximization of specific protein expression and product yield at the molecular level. In this study, the effect of varying molecular-size fractions of proteose peptone on α-amylase production in recombinant *Y. lipolytica* under the control of *XPR2* promoter was studied. It will be shown that use of active polypeptide fractions of proteose peptone in combination with a well-selected bioprocess strategy can significantly enhance the α-amylase productivity of recombinant *Y. lipolytica*.

MATERIALS AND METHODS

Recombinant Strains and Plasmids

A recombinant strain for α-amylase production was constructed *(17)*. The yeast strain used was *Y. lipolytica* SMY2 *(Aade1, ura3, xpr2)*, derived from *Y. lipolytica* CX161-1B *(Aade1)*, carrying an integrative vector pXOS103-In. Plasmid pOS103, which carries the rice α-amylase cDNA, was kindly provided by R. L. Rodriguez *(18,19)*. It contains the rice α-amy-

lase coding sequence and its signal peptide, the *XPR2* promoter and terminator of the gene encoding AEP of *Y. lipolytica*, and the *URA3* selective marker. The integrative vector was linearized at the *ClaI* site and integrated into the host chromosomal DNA using the lithium acetate transformation procedure *(20)*. Transformants were selected from uracil-free SD medium plate (glucose, 20 g/L; Bacto-yeast nitrogen base without amino acids, 6.7 g/L; adenine, 50 mg/L; Bacto-agar, 20 g/L). The integrated gene is very stable under nonselective conditions *(21)*.

Media

The media used in this work are summarized in Table 1. Culture medium of proteose peptone, agar, yeast nitrogen base without amino acids, and yeast nitrogen base without amino acids and ammonium sulfate were purchased from Difco (Detroit, MI). All other chemicals used were reagent-grade (Sigma, St. Louis, MO). To study the effect of active polypeptide fractions of proteose peptone on α-amylase production, the active polypeptide fractions were separated and used in the production medium. The amount of active polypeptide fractions contained in the production medium was equivalent to the amount of proteose peptone on the basis of total nitrogen content. The active polypeptide fractions were separated from the proteose peptone comprising 0.016 g total nitrogen/mL solution. One gram of proteose peptone contains 0.138 g of total nitrogen, as estimated by the Kjeldahl method.

Preparation of Active Polypeptide Fractions of Proteose Peptone

The flowchart for preparation of active polypeptide fractions of proteose peptone is shown in Fig. 1. A gel-filtration chromatography system (Pharmacia, Uppsala, Sweden), consisting of an XK 16/100 column equipped with Pharmacia LKB UV-MII monitor and p-1 pump, and a pilot scale BP 252/100 column equipped with dual-path monitor control optical unit and Cole-Parmer 7553-20 peristaltic pump were used to fractionate the active polypeptide fractions from proteose peptone. The gels used in this study were Sephacryl S-100 and coarse Sephadex G-25 (Pharmacia, Uppsala, Sweden). Approximately 2% bed volume (v/v) of proteose peptone solution (180 g/L) was loaded with Tris buffer (10 m*M*, pH 8.0) as a running buffer, unless otherwise specified. The buffer flow rates for XK 16/100 and BP 252/100 column were 0.45 and 350 mL/min, respectively.

A reverse osmosis (RO) system (Millipore, Bedford, MA), consisting of a Helicon-RO cartridge equipped with a NANOMAX membrane, was used to concentrate the diluted active polypeptide fractions obtained from the gel filtration of proteose peptone. The transmembrane pressure and temperature were maintained at 20 bar and 4°C, respectively.

Table 1
Media Compositions Used for This Study

Components	Batch	Fed-batch		Unit
	Growth	Growth	Production	
Glycerol	10	450	250	g/L
$(NH_4)_2SO_4$	—	72	—	g/L
Proteose peptone	—	—	100	g/L
Yeast nitrogen base w/o amino acid	3.4	—	—	g/L
Yeast nitrogen base w/o amino acids and ammonium sulfate	—	—	—	g/L
KH_2PO_4	—	13.5	7.5	g/L
$MgSO_4 \cdot 7H_2O$	—	5	3	g/L
Inositol	—	0.07	0.04	g/L
Thiamine	—	0.01	0.01	g/L
Trace metal solution[a]	—	40	25	ml/L
Vitamin solution[b]	—	40	25	ml/L
Adenine	50	1.5	1.0	g/L
Concentrated HCl	—	7	7	ml/L
Antifoam 289, Sigma	0.1	0.5	0.3	ml/L

[a]Trace metal solution (g/L): $FeCl_3 \cdot 6\ H_2O$–2.7, $ZnCl_2 \cdot 4H_2O$–2.0, $CaCl_2 \cdot 2H_2O$–2.0, $Na_2MoO_4 \cdot 2H_2O$–2.0, $CuSO_4 \cdot 5H_2O$–1.9, H_3BO_3–0.5, $MnSO_4$–2.0; concentrated HCl–10 ml/L.
[b]Vitamin solution (g/L): pantothenic acid—5.4, pyridoxine—1.4, niacin—6.1, folic acid—0.04, *d*-biotin—0.06.

Inoculum Preparation

A single colony isolate from an SD medium plate was used to inoculate 100 mL of growth medium in a 500-mL flask and was incubated in a rotary shaker (Hotech, 705R, Taipei, Taiwan) at 250 rpm and 28°C until the culture reached an OD_{600} of 1.0–1.5 within 17–24 h after inoculation. Then, this flask culture was used as an inoculum for the larger-scale cultivation.

Fed-Batch Cultures

Fed-batch cultures were conducted in a Chemap CBC5 6-L fermenter (Chemap, Switzerland). The seed culture (24 h old 100-mL shake-flask culture at OD_{600} of 1.5) was inoculated into the fermenter containing 1.5-L of growth medium. The temperature was maintained at 28 ± 0.1°C, aeration at 1–2 vvm, impeller speed at 600–900 rpm, and the pH at 6.8 ± 0.05 with 4 *N* NaOH. Dissolved oxygen was maintained above 25% saturation by supplying with oxygen-enriched air. The fed-batch culture began as a batch process, which was followed by a two-phase fed-batch cultivation.

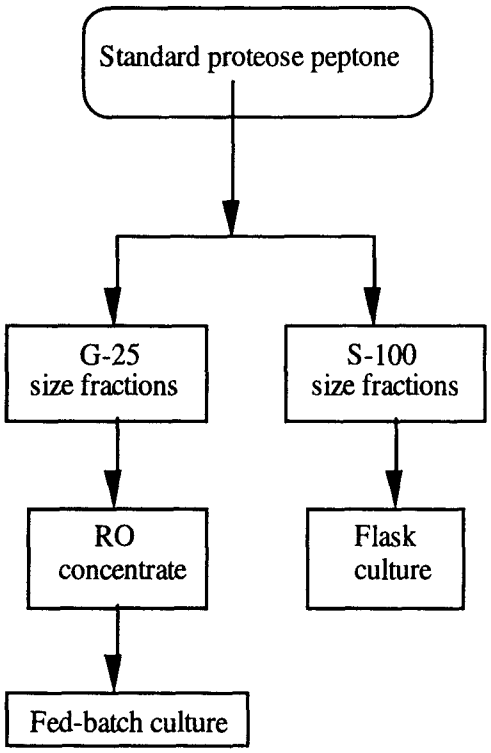

Fig. 1. Flowchart for preparation of active polypeptide fractions of protease peptone.

Glycerol was used as the limiting substrate. In the batch phase, when the glycerol was completely depleted, after 22–24 h batch cultivation, a peristaltic pump was activated and the growth medium was fed to the culture vessel. Once a sufficient biomass (20–25 g/L) was reached with the feeding of growth medium, this medium was switched to production medium to induce the rice α-amylase expression. To maintain a constant cell-growth rate, the theoretical feed rate based on time was derived from a cell and limiting substrate mass balance, represented by the following equation when S = 0 and dS/dt = 0:

$$F = [(\mu X_0 V_0)/(S_0 Y_{X/S})] \exp(\mu t) \quad (1)$$

where S is limiting substrate concentration in culture vessel, μ the preset specific growth rate, X_0 the initial cell concentration, V_0 the initial working volume, S_0 the limiting substrate concentration in the feeding medium, $Y_{X/S}$ the cell yield coefficient, and F the medium feed rate. In this study, we used a stepwise feeding strategy represented by the following semiempirical equation:

$$F = (1.5\ \mu\ X\ V)/(S_0\ Y_{X/S}) \quad (2)$$

where the coefficient, 1.5, is an empirical constant, X the cell concentration, and V the culture volume.

In all of the fed-batch experiments, the specific growth rate (μ) was set at 0.1 h^{-1}, and the cell yield coefficient ($Y_{X/S}$) was set at 0.5 for the growth medium and 0.52 for the production medium. The cell doubling time was 6.93 h, since the specific growth rate was set at 0.1 h^{-1}. The medium feed rate was adjusted approximately every 7 h according to Eq 2, and the specific growth rate was controlled within the range of 0.1 ± 0.02 h^{-1} based on the feeding strategy preprogrammed.

Cell Concentration Determination

Cell concentration (gram dry cell weight per liter) was measured with a Hitachi, 150-20 spectrophotometer (Tokyo, Japan) as optical density at 600 nm (OD_{600}) and converted to dry cell weight per liter of whole broth by using calibration data (1 OD_{600} unit = 0.52 g/L).

Analysis of Glycerol, Total Nitrogen, and α-Amylase

Whole-broth samples taken for the glycerol and α-amylase assays were centrifuged at 10,000 *Xg* for 5 min. The supernatants were stored at −20°C until analyzed.

The glycerol concentration was determined using a Jasco high-performance liquid chromatography (HPLC) system (Tokyo, Japan), consisting of 880-PU HPLC pump, 802-SC system controller, 851-AS sampler, and 830-RI refractive index detector equipped with a Bio-Rad Aminex HPX-87H column. The column was set up in an oven in which the temperature was maintained at 50°C. The mobile phase was a 5-m*M* sulfuric acid solution. The flow rate of the mobile phase was 0.6 mL/min. The peak heights were detected by refractive index detector.

The total nitrogen was measured by a Kjeltec system (Tecator, Hoganas, Sweden), consisting of 1026 distilling unit and digestion system.

The definition of an enzyme unit and the measurement of α-amylase have been reported previously (*22*). The reaction was initiated by adding 0.5 mL of enzyme solution (diluted if necessary) to 0.5 mL substrate solution (5 m*M* $CaCl_2$/50 m*M* sodium acetate buffer, pH 5.0, containing 1% soluble starch). After 10 min of incubation at 30°C, the reaction was terminated by adding 0.5 mL of 3,5-dinitrosalicylic acid solution and incubating in a boiling water bath for 5 min. The reaction was then cooled with running tap water and diluted with 4 mL of double-distilled water. The extent of product formation was monitored at A_{540} with a spectrophotometer (Hitachi, 150-20). Glucose was used as a standard. One unit of α-amy-

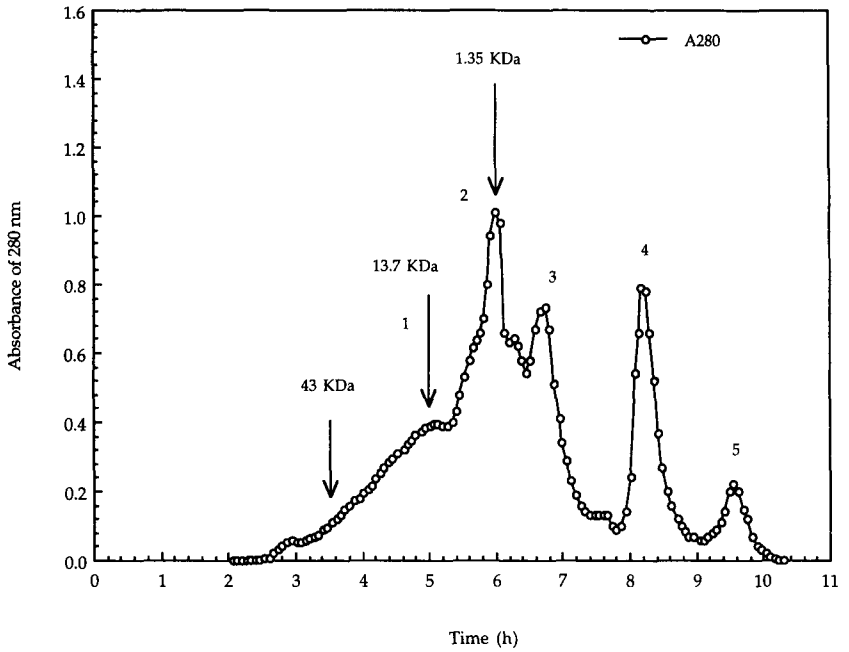

Fig. 2. Protein concentration profile of standard proteose peptone. (O) Absorbance at 280 nm. Column: XK 16/100, Sepharcyl-100. Flow rate: 0.45 mL/min. Running buffer: Tris, 10 mn, pH 8.0.

lase activity was defined as the amount of enzyme required to liberate 1 μmol of glucose/min.

RESULTS AND DISCUSSION

Effect of Active Polypeptide Fractions of Proteose Peptone on α-Amylase Production

With recombinant *Y. lipolytica* cultures, proteose peptone media containing low-mol-wt polypeptides have been used to induce cloned gene expression. Some components of the proteose peptone must have an inducer effect *(12,15)*. Figure 2 shows a fractionation profile of UV absorbance of A_{280} obtained from proteose peptone. The arrows in Fig. 2 indicate the molecular weight of the size fractions separated from the proteose peptone corresponding to the known mol-wt protein standards (ovalbumin, 43 kDa; RNase A, 13.7 kDa; cyanocobalamin, 1.35 kDa) used in this analysis. The effect of active polypeptide fractions of proteose peptone on rice α-amylase expression was investigated in shake flasks as shown in Fig. 3. The cell concentrations obtained using the proteose peptone fractions, which correspond to the molecular-size fractions designated by the peak

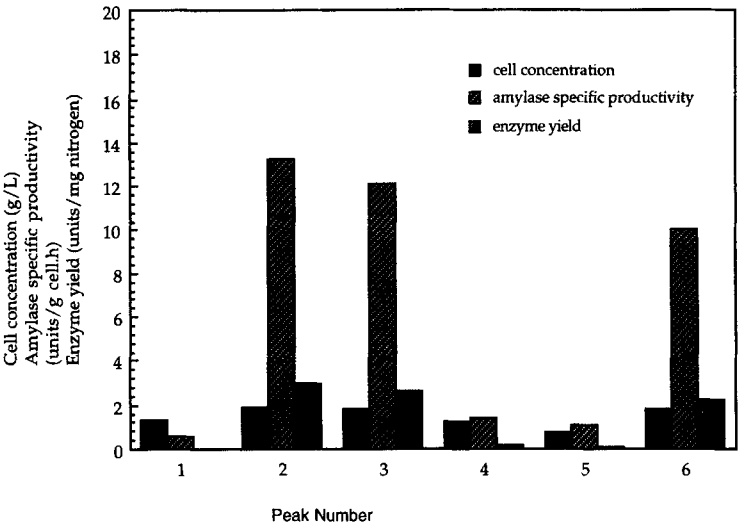

Fig. 3. Effect of active polypeptide fractions of proteose peptone on α-amylase expression in batch cultures. Peak numbers 1–5 correspond to those polypeptide fractions designated by the peak numbers in Fig. 2. Peak number 6 represents the control in which standard proteose peptone was used as a nitrogen source; (■) cell concentration; (▤, striped) α-amylase specific productivity; and (▨, shaded) enzyme yield.

numbers in Fig. 2, were similar among peaks 2, 3, and 6 (control) and somewhat lower for peaks 1, 4, and 5. The specific productivity of α-amylase (U/g cell·h) and the enzyme yield (U/mg nitrogen consumed) obtained with different polypeptide size fractions of proteose peptone were significantly different. The cell concentration of peak 1 was substantial, but the enzyme yield was negligible. This is partially attributable to the ammonium sulfate transferred from seed culture broth and acid protease production at low pH of culture broth. The final pH of culture broth was approx 5.2. Peaks 4 and 5 show that the enzyme specific productivity and α-amylase yield are lower than 1.39 (U/g cell·h) and 0.20 U/mg nitrogen consumed, respectively. These values are significantly lower than those shown by the peaks 2, 3, and 6. The active polypeptide fractions corresponding to the peaks 2 and 3 show an improvement of α-amylase specific productivity (13.21 and 12.14 U/g cell·h) and enzyme yield (2.98 and 2.64 U/mg nitrogen consumed) by 20–35.4% over the control, which corresponds to the peak 6 (10 U/g cell·h and 2.2 U/mg nitrogen consumed) for which the sole source of nitrogen was proteose peptone. These results indicate that only those polypeptide fractions corresponding to the peaks 2 and 3 increased α-amylase productivity in recombinant *Y. lipolytica* as compared to that obtained with the proteose peptone. The mol-wt range of those active polypeptide fractions are estimated to be 1.0–6.0 kDa.

Effect of Amino Acid on α-Amylase Induction and Production

Several researchers *(23–25)* have verified that the production of heterologous protein by recombinant *Bacillus brevis* was improved by amino acid supplement in the defined medium. When *Y. lipolytica* recombinant strain is grown in the production medium containing proteose peptone, little or no AEP is produced until the low-mol-wt polypeptide components are exhausted. Thereafter, the AEP production rate remains essentially constant over the logarithmic growth phase. It finally drops to zero in the stationary phase *(11)*. This observation suggests that after the free amino acids and low-mol-wt polypeptides are exhausted, the expression of the *XPR2* gene is induced by the active polypeptide fractions in the proteose peptone. Experimental results in Figs. 2 and 3 show that induction of heterologous protein expression by the polypeptide fractions of molecular weight < 1.0 kDa and higher than 6.0 kDa is insignificant. It was found that the proteose peptone contained all 20 amino acids (data not shown). The low-mol-wt fractions of proteose peptone containing all 20 amino acids (mol-wt size < 1000) were not an effective inducer for the α-amylase expression as shown in the previous section. In order to test the effectiveness of individual amino acids as inducers for α-amylase in recombinant *Y. lipolytica*, the production medium in which the proteose peptone was replaced by only one amino acid as a sole source of nitrogen was evaluated.

The effect of each amino acid on the induction of α-amylase expression was investigated in batch culture, and the results are shown in Table 2. The cell yield and α-amylase productivity were very low or negligible for most amino acids, except for glutamic acid and aspartic acid. Asparagine and histidine can induce α-amylase expression, but only glutamic acid and aspartic acid showed a significant cell growth and α-amylase expression as compared to the control. However, the α-amylase yield was lower when compared with the control containing proteose peptone.

Experimental results in Table 2 also suggest that those amino acids evaluated are not effective inducers for the *XPR2* promoter with the exception of asparagine, histidine, glutamic acid, and aspartic acid. The effects of amino acids on cell growth and α-amylase expression were investigated when the active polypeptide fractions of proteose peptone was supplemented with those individual amino acids as putative inducers that are partially effective for α-amylase expression. Each amino acid (5 m*M*) was added to the production medium containing the active polypeptide fractions of proteose peptone as the nitrogen source. The results of amino acid supplement are shown in Table 3. The cell yields were similar for all amino acids tested except for the cysteine supplemented medium, which gave a significantly lower cell yield as compared with all others. Enzyme-specific

Table 2
Cell Growth and α-Amylase Expression in Flask Culture Using an Amino Acid as the Sole Nitrogen Source

Amino acid	Cell yield, g/L[a]	α-Amylase yield u/mL[a]
Control[b]	1.86	0.60
Glycine	0.47	<0.03
Alanine	0.46	<0.03
Valine	0.36	<0.03
Leucine	1.05	<0.03
Isoleucine	0.25	<0.03
Proline	0.36	<0.03
Phenylalanine	0.28	<0.03
Tyrosine	0.29	<0.03
Tryptophan	0.11	<0.03
Serine	0.57	<0.03
Threonine	0.15	<0.03
Cysteine	0.26	<0.03
Methionine	0.37	<0.03
Asparagine	0.66	0.120
Glutamine	1.44	<0.03
Aspartic acid	2.28	0.24
Glutamic acid	2.41	0.240
Lysine	0.72	<0.03
Arginine	0.97	<0.03
Histidine	0.34	0.15

[a]The proteose peptone was substituted by an amino acid in the production medium 1.7 g/L of each amino acid was used in these experiments.

[b]The control represents the culture performance in the production medium.

productivity and α-amylase yield varied widely in comparison to the control. In the case of the cysteine-supplemented medium, the values of cell yield, enzyme-specific productivity, and α-amylase yield were the lowest. However, the supplementation with aspartic acid, glutamic acid, and histidine improved α-amylase expression by 10–53% above the control. This indicates that the supplementation of these three amino acids enhances both the cell growth and α-amylase expression in recombinant *Y. lipolytica*. Cysteine is the least effective amino acid in its enhancement of cell growth or α-amylase expression, whereas glutamic acid is the most effective.

Table 3
Effects of Individual Amino Acids on Cell Growth, Cell Specific Activity, and α-Amylase Yield in Flask Cultures with Active Polypeptide Fractions of Proteose Peptone as the Nitrogen Source

Amino acid	Cell yield, g/L[a]	Specific productivity, u/g cell·h[a]	α-Amylase yield, u/mg nitrogen consumed[a]
Control[b]	2.03	14.75	3.57
Glycine	2.34	9.04	2.52
Alanine	2.48	7.93	2.35
Valine	2.26	9.46	2.55
Leucine	2.12	6.36	1.61
Isoleucine	2.24	6.25	1.67
Proline	2.50	7.14	2.13
Phenylalanine	1.84	7.68	1.69
Tyrosine	2.04	8.50	2.07
Tryptophan	1.98	13.20	3.12
Serine	2.50	13.40	4.00
Threonine	2.26	7.96	2.15
Cysteine	1.43	3.39	0.58
Methionine	2.50	5.57	1.66
Asparagine	2.31	13.07	3.60
Glutamine	2.09	8.75	2.18
Aspartic acid	2.08	16.25	4.03
Glutamic acid	2.19	20.93	5.47
Lysine	2.50	9.54	2.85
Arginine	2.48	6.46	1.91
Histidine	2.06	17.64	4.44

[a]The proteose peptone was substituted by active polypeptide fractions of proteose peptone in the production medium, 5 m*M* of each amino acid were added to the production medium.

[b]The control represents the culture performance in the production medium, in which the proteose peptone was substituted by active polypeptide fractions of proteose peptone as a nitrogen source.

Fed-Batch Culture Experiments

Effect of the Glycerol/Proteose Peptone Ratio (C/N) on α-Amylase Productivity

The effect of ratio (C/N) of glycerol to proteose peptone on α-amylase production with recombinant *Y. lipolytica*, which is under the control of the *XPR2* promoter, was investigated in fed-batch culture. Experimental

Table 4
Summary of Fed-Batch Cultures of *Y. lipolytica* with Various Nitrogen Sources and the C/N Ratios of Glycerol/Proteose

Nitrogen source	C/N ratio	Cell conc. g/L	α-Amylase yield, U/mL	α-Amylase specific productivity, u/g cell·h	Enzyme yield, u/mg nitrogen	Consumed total nitrogen g	Data Ref.
Standard proteose peptone	1/0.34	69.3	24.9	25.7	4.96	10.8	
Standard proteose peptone	1/0.4	64.0	28.5	30.7	5.19	13.61	
Active polypeptide fractions of proteose peptone	1/0.34	66.9	31.9	33.0	6.73	10.78	Fig. 4
Active polypeptide fractions of proteose peptone	1/0.4	66.0	16.0	20.4	3.21	10.98	
Active polypeptide fractions of peptone plus glutamic acid (28 g/L)	1/0.34	63.0	40.0	36.2	8.14	12.29	Fig. 5

[a]Figure and data are not shown. The profile trends are similar to Fig. 4.
[b]Figure and data are not shown. The profile trends are similar to Fig. 5.

results are summarized in Table 4. After induction, α-amylase production continued at a constant rate during the production phase. The final cell concentration and α-amylase activity attained were 69.3 g/L and 24.9 U/mL with C/N ratio of 1/0.34, and 64.0 g/L and 28.5 U/mL with C/N ratio of 1/0.4, respectively. The α-amylase-specific productivity (30.7 U/g cell·h) with C/N ratio of 1/0.4 was approx 20% higher than that (25.7 U/g cell·h) with a C/N ratio of 1/0.34. When proteose peptone was used as a nitrogen source, ammonium ion was generated by the microorganisms. Chang et al. *(23)* reported that α-amylase specific activity was decreased as the C/N ratio further decreased to 1/1.67 in the culture medium. The decrease in enzyme-specific productivity was owing to ammonium ion repression. This indicates that the C/N ratio plays an important role in the overproduction of α-amylase from recombinant *Y. lipolytica.*

α-Amylase Production Using Active Polypeptide Fractions of Proteose Peptone

Recognizing the effect of active polypeptide fractions of proteose peptone on α-amylase expression (Fig. 3), the effect of ratio of glycerol to active polypeptide fraction in a fed-batch culture was also investigated.

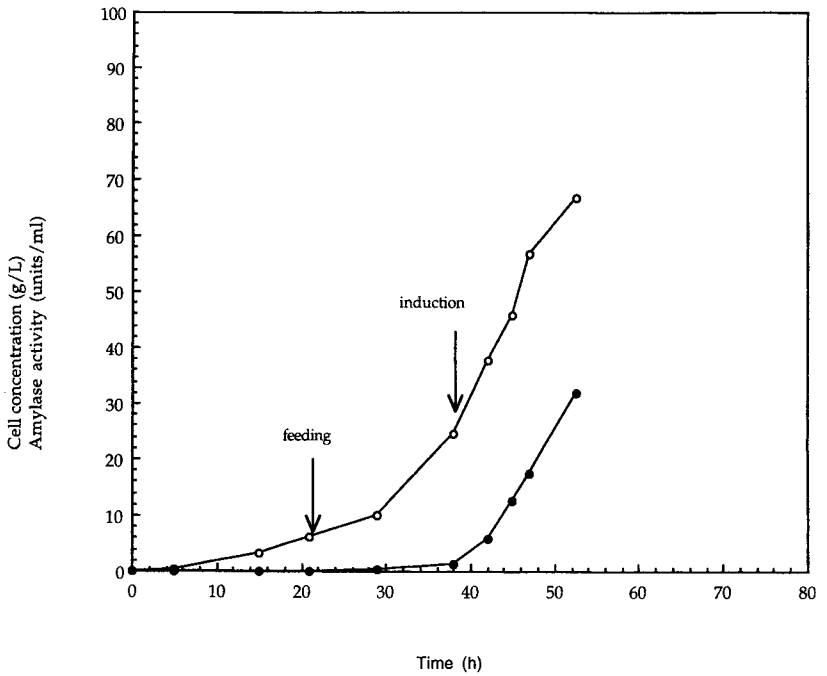

Fig. 4. Time-course profiles of cell concentration and α-amylase activity in fed-batch culture. The active polypeptide fractions of proteose peptone was used as a nitrogen source on production medium. The ratio of glycerol to active polypeptide fractions of proteose peptone was 1/0.34. (○) Cell concentration; (●) α-amylase activity.

The final cell concentration and α-amylase activity attained were 66.9 g/L and 31.9 U/mL with C/N ratio of 1/0.34, and 66.0 g/L and 16.0 U/mL with C/N ratio of 1/0.4 respectively. The specific productivity (33 U/g cell·h) with C/N ratio of 1/0.34 is approx 1.6 times higher than that of C/N ratio of 1/0.4 (20.4 U/g cell·h) (Table 4 and Fig. 4). The ammonium ion concentration is practically zero during the production phase (data not shown). Ogrydziak et al. *(5)* reported that an ammonium ion pool at a very low concentration (<10 mmol/g dry cell weight) of AEP production was derepressed in wild *Y. lipolytica*. The decreasing trend in the specific productivity of α-amylase as a result of decreasing C/N ratio may be attributed to the high ammonium ion pool inside the cells when active polypeptide fractions of proteose peptone are used.

Enhancement of α-Amylase Production with Glutamic Acid-Enriched Active Polypeptide Medium

Recognizing the effect of glutamic acid on α-amylase induction and production, a fed-batch culture with the feeding of glutamic acid-enriched production medium containing active polypeptide fractions was investi-

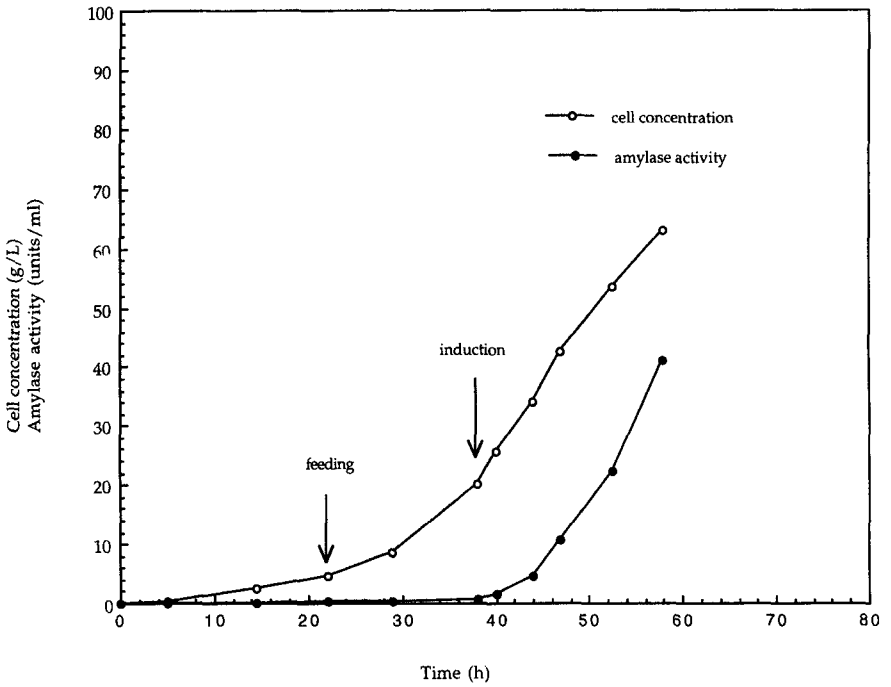

Fig. 5. Time-course profiles of cell concentration and α-amylase activity in fed-batch culture. The ratio of glycerol to active polypeptide fractions of proteose peptone was 1/0.34. Approximately 28 g/L of glutamic acid was supplemented to the production medium. (○) Cell concentration; (●) α-amylase activity.

gated. The glutamic acid concentration in the production medium was 28 g/L. Experimental results are shown in Fig. 5. Cell yield was similar to that obtained using proteose peptone medium. The α-amylase specific productivity was significantly higher when the glutamic acid-enriched active polypeptide medium was used as compared with the control (Table 4). The final cell concentration and α-amylase specific productivity attained were 63 g/L and 36.2 U/g cell·h, respectively. The specific productivity of α-amylase and enzyme yield were improved by 41 and 64%, respectively, as compared with the results obtained with the medium containing proteose peptone at C/N ratio of 1/0.34 (Table 4 and Fig. 5).

CONCLUSIONS

The polypeptide fractions of proteose peptone that are effective for induction of α-amylase expression in recombinant *Y. lipolytica*, which is under the control of *XPR2* promoter, have a range of molecular size, 1.0 and 6.0 kDa. The specific productivity of α-amylase and enzyme yield

vary with differing nitrogen sources and C/N ratios. The fed-batch bioprocess strategy in combination with a rational medium design and the proper selection of both the nitrogen source and C/N ratio are very important to the gene expression and productivity of heterologous protein in recombinant *Y. lipolytica.*

A significant α-amylase productivity enhancement by the use of glutamic acid-enriched medium containing the active polypeptide fractions of proteose peptone was observed, although the regulatory effect of glutamic acid on the enhancement of heterologous protein productivity has not been completely elucidated. Nevertheless, the use of glutamic acid-enriched culture medium containing active polypeptide fractions of proteose peptone as a nitrogen source in fed-batch culture is a very effective bioprocess strategy to overexpressing other heterologous proteins using *Y. lipolytica* recombinant.

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REFERENCES

1. Lee, S. B., Ryu, D. D. Y., Siegel, R., and Park, S. H. (1988), *Biotechnol. Bioeng.* **31,** 805–820.
2. Raibound, E., Stanssens, P., and Fiers, F. W. (1981), *Gene* **15,** 81–93.
3. Yabuta, M., Onai-Miura, S., and Ohsuye, K. (1995), *J. Biotechnol.* **39,** 67–73.
4. Couderc, R., and Baratti, J. (1980), *Agric. Biol. Chem.* **44,** 2279–2289.
5. Ogrydziak, D. M., Demain, A. L., and Tannenbaum, S. R. (1977), *Biochim. Biophys. Acta* **497,** 528–535.
6. Blondeau, K., Boutur, O., Boze, H., Jung, G., Moulin, G., and Galzy, P. (1994), *Appl. Microbiol. Biotechnol.* **41,** 324–329.
7. Patkar, A. and Seo, J. H. (1992), *Biotechnol. Bioeng.* **40,** 103–109.
8. Hoffman, B. J., Broadwater, J. A., Johnson, P., Harper, J., Fox, B. G., and Kenealy, W. R. (1995), *Protein Expression and Purification* **6,** 646–654.
9. Vedvick, T., Buckholz, R. G., Engel, M., Urcan, M., Kinney, J., Provow, S., et al. (1991), *J. Ind. Microbiol.* **7,** 197–201.
10. Reiser, J., Glumoff, V., Kalin, M., and Ochsner, U. (1990), *Adv. Biochem. Eng. Biotechnol.* **43,** 75–102.
11. Davidow, L. S., O'Donnel, M. M., Kaczmarek, F. S., Pereirra, D. A., DeZeeuw, J. R., and Franke, A. E. (1987), *J. Bacteriol.* **169,** 4621–4629.
12. Nicaud, J. M., Fournier, P., La Bonnardiere, C., Charles, M., and Gaillardin, C. (1991), *J. Biotechnol.* **19,** 259–270.
13. Tharaud, C., Ribet, A. M., Costes, C., and Gaillardin, C. (1992), *Gene* **121,** 111–119.

14. Franke, A. E., Kaczmarek, F. S., Eisenhard, M. E., Geoghegan, K. F., Danley, D. E., DeZeeuw, J. R., et al. (1988), *Dev. Ind. Microbiol.* **29,** 43–57.
15. Chang, C. C. (1997), PhD thesis. University of California, Davis.
16. Le Dall, M. T., Nicaud, J. M., and Gaillardin, C. (1994), *Curr. Genet.* **26,** 38–44.
17. Park, C. S., Chang, C. C., Kim, J. Y., Ogrydziak, D. M., and Ryu, D. D. Y. (1997), *J. Biol. Chem.* **272**(11), 6876–6881.
18. Kumagai, M. H., Shah, M., Terashima, M., Vrkljan, Z., Whitaker, J. R., and Rodriguez, R. L. (1990), *Gene* **94,** 209–216.
19. O'Neill, S. D., Kamagai, M. H., Majumdar, A., Huang, N., Sutliff, T. D., and Rodriguez, R. L. (1990), *Mol. Gen. Genet.* **221,** 235–244.
20. Ito, H., Fukuda, Y., Murata, A., and Kimura, A. (1983), *J. Bacteriol.* **153,** 163–168.
21. Chang, C. C., Ryu, D. D. Y., Park, C. S., Kim, J. Y., and Ogrydziak, D. M. (1998), *Appl. Microbiol. Biotechnol.* **49,** 531–537.
22. Bernfeld, P. (1955), *Methods Enzymol.* **1,** 149–158.
23. Chang, C. C., Ryu, D. D. Y., Park, C. S., and Kim, J. Y. (1998), *Biotechnol. Bioeng.* **59,** 379–385.
24. Park, Y. S., Dohjima, T., and Okab, M. (1995), *Biotechnol. Bioeng.* **49,** 36–44.
25. Miyashiro, S., Enei, H., Hirose, Y., and Udaka, S. (1980), *Agricultural Biol. Chem.* **44,** 105–112.