

Production and recovery of recombinant protease inhibitor α_1 -antitrypsin

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Abstract

Human α_1 -antitrypsin (AAT) was produced in the recombinant yeast *Saccharomyces cerevisiae* ATCC 20699 grown in batch and fed-batch culture. The final biomass concentration and antitrypsin concentration attained were $\sim 55 \text{ g}\cdot\text{L}^{-1}$ and $1.23 \text{ g}\cdot\text{L}^{-1}$, respectively, in the fed-batch. The maximum productivities of biomass and antitrypsin were 1.6 and $> 0.04 \text{ g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$, respectively, or substantially greater than the highest productivity values reported in the past. For recovering the antitrypsin, the cell slurry was concentrated 4-fold ($231 \text{ g}\cdot\text{L}^{-1}$ biomass, 122 min of processing) by cross-flow microfiltration and the cells were disrupted by bead milling (3 passes of 3 min total retention time). The cell homogenate was treated with aluminum chloride or PBS (pH 7) to aid separation of the cell debris by flocculation and sedimentation. The clarified cell homogenate was subjected to ammonium sulfate fractionation to precipitate the recombinant antitrypsin. The AAT precipitated at 45–75% saturation of ammonium sulfate, depending on the age of the homogenate. The crude AAT in the homogenate degraded at room temperature (25°C), with a zero order deactivation rate of $1.815 \times 10^{-3} \pm 3.43 \times 10^{-4} \text{ g AAT L}^{-1}\cdot\text{h}^{-1}$. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Recombinant *Saccharomyces cerevisiae*; α_1 -antitrypsin; Protease inhibitor; Cell disruption

1. Introduction

This work details the production and recovery of human α_1 -antitrypsin (AAT) from the recombinant yeast *Saccharomyces cerevisiae* ATCC 20699. α_1 -Antitrypsin is a blood protein that inhibits serine proteases. AAT is a major protease inhibitor in human plasma and its primary physiological role is to inhibit neutrophil elastase [19]. In the event of AAT insufficiency, elastase attacks and damages lung tissue, causing potentially fatal emphysema, especially among smokers. AAT deficiency is genetically determined, but inhalation of environmental pollutants, including tobacco smoke, can lower AAT activity [16]. Human emphysematous condition is said to be relieved by i.v. injection of partially purified α_1 -antitrypsin [4]. AAT administration is beneficial also in some cases of cystic fibrosis. AAT for augmentation therapy is currently purified from donor blood

but this source is limited and prone to contamination with infectious agents [7,19,20].

Antitrypsin purified from blood plasma is commercially sold as Prolastin[®] (Bayer Corp.). This product is expensive (approx. \$130/g). Patients with hereditary deficiency of α_1 -antitrypsin are given a dose of 120–180 mg AAT per kg of body weight every two to three weeks [1]. The high cost of blood-derived AAT results from several factors, including a low concentration of AAT in blood; a complex purification protocol; and tests and processes that are needed to provide a reasonable degree of assurance of the product's freedom from blood-borne diseases. Purification of the α_1 -antitrypsin from blood is a complex process requiring removal of albumin by chromatography; ammonium sulfate fractionation; DEAE-cellulose chromatography; and ultrafiltration [25]. Other purification approaches have included initial precipitation of contaminating plasma proteins prior to the DEAE-cellulose chromatography step [14]. Glutathione-sepharose column chromatography has also been used in combination with other steps [23].

A less expensive supply of AAT from a recombinant producer can greatly reduce the cost of treating patients.

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Human recombinant AAT has been expressed in various yeasts [4,9,17–19], the bacterium *Escherichia coli* [2,20], animal cells [26], and plant cells [29,30]. Plant and animal cells generally produce low levels of AAT; higher productivities can be obtained in yeasts and bacteria. However, production in bacteria is fraught with significant problems: 1. The protein is produced as denatured inclusion body and recovery is costly [7]. 2. The recombinant protein is not glycosylated and this can affect performance in the intended application. [For example, compared with the nonglycosylated AAT, the native glycosylated AAT is more stable against thermal denaturation and denaturation by urea [19, 21]. 3. Bacteria such as *E. coli* are non-GRAS (Generally Regarded as Safe) species. 4. Proteins produced in bacteria are routinely contaminated with cell wall derived endotoxins and must be purified rigorously [7]. Because of these factors, recombinant yeasts are the preferred production vehicles for therapeutic proteins in general and for AAT.

Although AAT has been expressed in several recombinant bacteria and yeasts [2,4,9,17–20], no high cell density culture has been reported for any AAT-producing microorganism. Also, the maximum final amount of the active AAT reported for any recombinant yeast culture has been low, at $75 \text{ mg}\cdot\text{L}^{-1}$ [17]. The work described here attained a maximum antitrypsin concentration of $1.23 \text{ g}\cdot\text{L}^{-1}$ in fed-batch culture and the highest attained productivity of antitrypsin exceeded $0.045 \text{ g}\cdot\text{L}^{-1}\text{h}^{-1}$. Significantly, production was attained in a GRAS yeast. Recombinant *S. cerevisiae* with different expression vectors [9,17] than used here have given much lower specific production of AAT than in the present work.

The intracellularly produced AAT was recovered by separation and disruption of the biomass; treatment of the homogenate with aluminum chloride or PBS (pH = 7) to enhance flocculation or sedimentation; and precipitation of the α_1 -antitrypsin with ammonium sulfate. The mass ratio of α_1 -antitrypsin to the total protein in the precipitate was about 0.13, comparable to a ratio of 0.16 obtained with ammonium sulfate/protamine sulfate fractionation of AAT produced extracellularly by *Saccharomyces diastaticus* [19].

2. Materials and methods

2.1. Microorganism and fermentation conditions

Human α_1 -antitrypsin was produced in the genetically engineered yeast, *Saccharomyces cerevisiae* ATCC 20699. The recombinant cell was derived from *S. cerevisiae* strain E 18 host (ATCC 20743). The vector for expression of mutant antitrypsin (AT) genes in the yeast was the c1/1 derivative pFATPOT. The vector pFATPOT comprised the amp[®], Leu 2 and 2 micron regions of c1/1; an expression unit composed of the *S. cerevisiae* triose phosphate isomerase (TPI) promoter, the wild type AT sequence from pUC

alpha 1, and the *S. cerevisiae* transcription terminator; and the *Schizosaccharomyces pombe* triose phosphate isomerase (POT 1) gene [16]. When transformed into a yeast host (such as *S. cerevisiae* ATCC 20743) defective in triose phosphate isomerase production, the POT 1 gene on the plasmid complements the host cell defect and allows for plasmid maintenance at high copy number during growth on rich media [16].

Shake flask experiments were done in 4 L flasks with 1 L medium of glucose (20 g), peptone (20 g), and yeast extract (10 g). The same medium was used to grow the inoculum. The inoculum size was 100 ml. The flasks were incubated in a temperature controlled (30°C) shaker (INNOVA 4330; New Brunswick Scientific Co., Inc., New Brunswick, NJ, USA) at 180 rpm agitation speed. The culture typically lasted 24 h.

For batch and fed-batch fermentations in the bioreactor, two staged inocula were grown in shake flasks as specified above. For the first stage, a loopful of slant culture was inoculated into a 500 ml flask containing 100 ml medium. This preculture was used to inoculate a 4 L flask with 1 L medium. Fermentations were carried out in a 15 L (nominal) stirred tank fermenter (MBR Bioreactor AG, Switzerland) equipped with controllers for pH, temperature, and dissolved oxygen. Presterilized medium (3 L) at 30°C, pH 4.0–4.5, was inoculated with 1 L inoculum described earlier. The fermentation medium contained (per liter) the following components: 35 g glucose, 25 g corn steep liquor, 10 g ammonium sulfate, 7.5 g peptone, 5 g yeast extract, 5 g potassium sulfate, 0.5 g calcium chloride, 0.5 g sodium chloride, 0.3 g magnesium sulfate-seven-hydrate, 20 ml of a trace elements solution, 10 ml supplemental solution, and antifoam (1000 ppm) (SIGMA, USA, cat. no. A 8436). The details of the trace elements solution and the supplemental solution have been published previously [10].

The bioreactor cultures were carried out in batch and fed-batch modes, always at 30°C. In batch cultures, the pH was controlled at 4.4 using 20% w/v aqueous ammonia. The impeller rotational speed was 500–600 rpm and the aeration rate was set at 2 vvm. These conditions produced actively growing cell mass at up to $15\text{--}17 \text{ gDW}\cdot\text{L}^{-1}$. Fed-batch operations commenced with an initial volume of 4 L. The feed composition was as follows (per liter): 260 g glucose, 9.5 g peptone, 12.5 g ammonium sulfate, 6.25 g potassium phosphate, 4.5 g yeast extract, 0.625 g magnesium sulfate-seven-hydrate; the other components were the same as specified for batch fermentation.

The feed and the ammonia solutions were fed from separate reservoirs. Approximately 4 L of feed and 300 ml of ammonia solution were used in the fermentation. The feeding was controlled in response to pH and dissolved oxygen (DO) concentration both of which tended to increase as the nutrients in the fermentation broth depleted. During fed-batch operation, the pH was maintained at 4.6 using the earlier specified ammonia solution. The rate of feeding was computed as detailed in the past [11] and

controlled manually. The agitation speed increased gradually from 600 rpm to 950 rpm so as to maintain the DO concentration above 20% of air saturation. The aeration rate was held constant at 2 vvm.

2.2. Biomass concentration

The yeast biomass in the fermentation broth was quantified either by dry-cell weight analysis or by measurement of optical density of the broth. For dry weight determinations, the cells were recovered by centrifugation (5500-g, 15 min; IEC Centra-HN centrifuge, International Equipment Company, Needham Heights, MA, USA) and washed once with deionized water. The recovered biomass paste was placed in pre-weighed aluminum dishes and dried to constant weight in an oven (80°C). For optical density measurements, the absorbance was read at 600 nm (PYE Unicam SP6–550 UV/VIS spectrophotometer, Philips Scientific and Analytical Equipment). The biomass concentration X ($\text{g}\cdot\text{L}^{-1}$) and the absorbance were related as follows:

$$X = 0.45 \times \text{Absorbance.} \quad (1)$$

2.3. Antitrypsin assay

Antitrypsin was determined by the elastase inhibition assay of Insley and Kawasaki (1987) [16], modified as follows. To a 50–200 μl pre-filtered (0.2 μ Gelman Sciences Filters, Bulk Supor Acrodisc, MI, USA) sample containing antitrypsin, 50 μl of 1 μg per microliter porcine pancreatic elastase (Sigma Chemicals Co, St Louis, MO, USA; cat. no. E-1250) in 0.2 M Tris (pH 8.8) was added. PBS was added to make up the volume to 1 ml. The resulting mixture was incubated at 40°C (15 min). 1 ml of 10 milligram per milliliter elastin-orcein (Sigma Chemicals Co, St Louis, MO, USA, cat. no. E 1500) in 0.4 M Tris (pH 8.8) was then added and incubation was continued for a further 60 min at 37°C, with mixing at 15 min intervals. The mixture was centrifuged and the absorbance of the supernatant was measured at a wavelength of 590 nm (Spectronic Genesys 2 spectrophotometer, Milton Roy Company NY, USA). The absorbance was measured against a blank which contained elastase dissolved in Tris buffer. A calibration curve was made for the 0.0–0.9 $\text{mg}\cdot\text{mL}^{-1}$ concentration range, using purchased α_1 -antitrypsin (Sigma Chemicals Co, St Louis, MO, USA, cat. no. A 6150). The absorbance values of the samples were subtracted from the absorbance (590 nm) value of the totally hydrolyzed orcein-elastin and the resulting corrected absorbance was correlated with the antitrypsin concentration according to the following calibration relationship:

$$\begin{aligned} \text{Antitrypsin } (\text{g}\cdot\text{mL}^{-1}) &= 1.81 \times 10^{-4} \\ &+ 1.757 \times 10^{-3} \times \text{Absorbance.} \end{aligned} \quad (2)$$

2.4. Stability of antitrypsin

For determinations of stability of the crude preparation, the antitrypsin-containing supernatant obtained from the filtered homogenate of the disrupted cells was sterile filtered (Sterile MILLEX_GV 0.22 μm durapore membrane filter, MILLIPORE, Bedford, MA, USA) into pre-sterilized test tubes and incubated for up to 9 h at 25°C. The quantity of antitrypsin in the tubes was measured after various periods of incubation, using the elastase inhibition assay.

2.5. Total protein

The Coomassie blue method was used for determining the total protein [3,28]. A 0.1 ml aliquot of the unknown dilute protein sample was pipetted into a test tube, 5.0 ml of Coomassie protein assay reagent (Pierce, Rockford, IL; cat. no. 23200) was added, and the resulting solution was thoroughly mixed. Absorbance of the mixture was read within 90 min at 595 nm (PYE Unicam SP6–550 UV/VIS spectrophotometer, Philips Scientific and Analytical Equipment) against a deionized water blank. The absorbance reading was converted to protein concentration using a standard curve and the known dilution factor. The standard curve was made by measurements on known solutions of bovine serum albumin Fraction V (Pierce, Rockford, IL; cat. no. 23210) in the 0–1000 $\mu\text{g}\cdot\text{mL}^{-1}$ concentration range. The measurements were not affected by ammonium sulfate [31].

2.6. Ethanol

Ethanol was assayed by gas chromatography (Hewlett-Packard, Avondale, PA, USA, Model 5880A chromatograph, equipped with flame ionization detector and 2 ft by 1/8 inch Porapak type T packing). The carrier gas was helium (20 $\text{ml}\cdot\text{min}^{-1}$ flow rate). The supernatant of the centrifuged (5500-g, 15 min) broth sample was used for ethanol measurements. Ethanol was quantified by comparison with a calibration curve that was made using ethanol solutions of known concentration (0–16 $\text{g}\cdot\text{L}^{-1}$).

2.7. Glucose

Glucose was analyzed by HPLC (Waters 700 Satellite WISP, Massachusetts, USA) on a RCM monosaccharide column (Rezex HPLC columns for carbohydrate and organic acid analysis). The mobile phase was double distilled water, at a flow rate of 0.5 $\text{ml}\cdot\text{min}^{-1}$. A refractometer was used for detection. The column temperature was maintained at 90°C. Standard glucose samples were prepared by dissolving glucose (1–20 $\text{g}\cdot\text{L}^{-1}$) in distilled water. The fermentation broth supernatant remaining after centrifugation (see Section 2.2) was filtered (0.2 μ Gelman Sciences Filters, Bulk Supor Acrodisc, MI, USA) prior to analysis.

2.8. Recovery of biomass by microfiltration

A Mini-DMF rotary microfiltration unit (Pall Trinity Micro, NY, USA) was used to recover the biomass from the broth. A similar filter has been described by Lee et al. [22]. The filter membrane was made of Nylon 66 and had a pore size of 0.45 μm . The filter area was 303 cm^2 . A high-speed solid plastic disc rotated (1000 rpm) near the surface of the membrane, to generate turbulence. The gap between the disc and the membrane was 0.5 cm and the diameter of the rotor was 0.15 m. An initial transmembrane pressure of 5 psi was used. The initial biomass concentration fed to the system was about 52–55 $\text{g}\cdot\text{L}^{-1}$, but the slurry became more concentrated with time of processing. The cell recovery operation was performed at 25°C.

2.9. Cell disruption and homogenate clarification

The cells were disrupted mechanically to release the intracellular antitrypsin. For small volumes (1–10 ml) of slurry, the broth was centrifuged at 5500-g (15 min) in a laboratory centrifuge (IEC Centra-HN, International Equipment Company, Needham Heights, MA, USA). The resulting pellet was resuspended in 2.3 ml of PBS and vortexed with glass beads (5 g, 425–600 μm bead diameter) (Sigma Chemical Co, St Louis, MO, USA; cat. no. G9268) for 5 min for disruption. The homogenate was centrifuged (9000-g, 15 min), filtered (0.2 μm Gelman Sciences Filters, Bulk Supor Acrodisc, MI, USA) and analyzed for α_1 -antitrypsin.

For larger quantities, the broth was concentrated by microfiltration in a rotary filter and washed with PBS. The washed cell slurry was concentrated (microfiltration) to attain an approximate biomass concentration of 231 $\text{gDW}\cdot\text{L}^{-1}$. For disruption, the concentrated slurry was diluted with PBS to about 170 $\text{gDW}\cdot\text{L}^{-1}$ and processed through a continuous flow bead mill (Sulzer Annu Mill 01, Sulzer Brothers Limited, Winterthur, Switzerland), as used previously by Garrido et al. (1994) [13] for disintegration of the yeast *S. cerevisiae*. The mill had been loaded with lead-free soda lime silica glass beads (425–600 μm bead diameter, 2,740 $\text{kg}\cdot\text{m}^{-3}$ density) to nominally occupy 85% of the volume of the grinding chamber (210 ml). The concentric cylindrical rotor in the grinding chamber rotated at 2,000 rpm to agitate the beads. The flow rate of the slurry through the mill was 100 $\text{ml}\cdot\text{min}^{-1}$. The temperature was controlled at 10–12°C by circulating cooling water in the jacket that surrounded the grinding chamber.

The effluent from the mill was reprocessed through the grinding chamber for a total of three to four passes, giving a cumulative disruption time of 2.9–3.9 min. Generally, three passes through the grinding chamber were sufficient for complete disruption. The disrupted slurry was held at 4°C and used as needed. After disruption, the pH of the homogenate was 5.6–5.8. In some cases, the pH was raised

to pH 7 by adding sodium hydroxide before further processing of the homogenate.

The homogenate was clarified of cell debris in one of two ways: Either a few drops of a saturated solution of aluminum chloride 6-hydrate were added to the homogenate (80 ml) with gentle stirring to attempt to flocculate the debris, or the homogenate was made to three times the original volume with PBS in attempts to facilitate sedimentation of the suspended solids. In both cases, the treated homogenate was centrifuged (5000-g, 15 min) to remove the solids.

2.10. Antitrypsin fractionation

Five equal portions (10 ml each) of the clarified crude cell homogenate were placed in separate beakers held on ice-baths. The pH of the crude homogenate was 5.6–5.8 but in some experiment the pH was raised to 7.0 (concentrated sodium hydroxide) prior to salt fractionation. Ammonium sulfate was added to beakers to obtain 15, 30, 45, 60, and 75% (w/v) concentration of the salt. During additions, the solution in the beakers was stirred using a magnetic stirring bar. Agitation was continued for 1 h. The precipitate formed was removed by centrifugation (10,000-g, 15 min, 4°C; Damon/IEC Division IEC B-20A centrifuge, International Equipment Company, Needham Heights, MA, USA). The precipitate samples were redissolved quantitatively in PBS buffer (pH 7.0), to produce the same volume of solution as the original samples of the homogenate. The resulting solutions were assayed for total protein and antitrypsin. Both protein and α_1 -antitrypsin were expressed, respectively, as g protein and g antitrypsin per liter of the original crude homogenate.

3. Results and discussion

3.1. Production of biomass and AAT

A typical fermentation profile obtained in shake flasks is shown in Fig. 1. Compared to culture in shake flask and batch fermentor, the biomass density attained in the fed-batch fermentation was up to 6-fold greater (Table 1). The maximum concentration of AAT in the fed-batch culture was nearly 8-fold greater than in the shake flask (Table 1). Although the shake flask cultures used the supposedly 'rich' medium recommended by Insley et al. (1987) [16] for enhancing production of α_1 -antitrypsin, the results were much poorer than obtained in the controlled environments of the fermenters (Table 1). The media used in the batch and fed-batch fermentations were identical but different from that of Insley et al. [16]. Fed-batch culture performed best (Table 1). Clearly, controlled cultures in the fed-batch mode are preferable to uncontrolled batch fermentations for commercially feasible production of AAT. The data in Table 1 were obtained from four batch and fed-batch fermentations and three shake flask cultures.

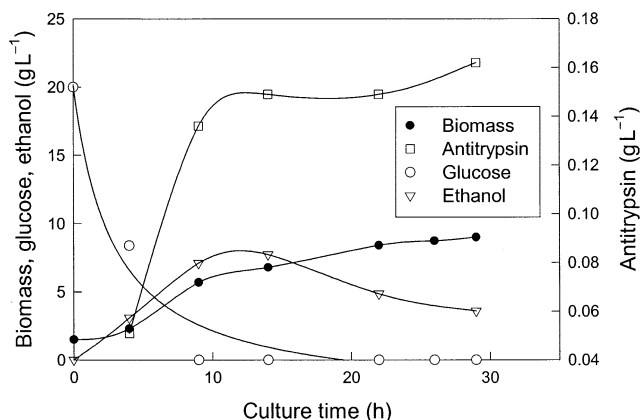


Fig. 1. Antitrypsin fermentation profile (shake flask culture).

Production of recombinant proteins is often associated with cell growth (e.g. Fig. 1) and attaining a high protein productivity requires a high cell density culture. Obtaining high biomass densities in yeast fermentation necessitates fed-batch culture [10,11]. This is because a carbon build-up causes a switch from highly productive oxidative metabolism to less productive fermentative production of ethanol [10,11]. The concentration of the carbon source is also known to affect the expression of the recombinant protein [6,15]. The need to maximize cell growth rate while suppressing ethanol production recommended fed-batch operation as the preferred culture strategy.

The best results so far reported in the literature for producing α_1 -antitrypsin were obtained in high cell density cultures of *S. cerevisiae* 2805 [17]. The authors attained a maximum biomass concentration of 28 g·L⁻¹ and the corresponding concentration of AAT was 0.075 g·L⁻¹ [17]. In contrast, because of the high specific productivity of AAT in *S. cerevisiae* ATCC 20699, we obtained more than twice as much AAT in the uncontrolled shake flasks (Table 1) as did Kang et al. [17]. The AAT concentration attained in our fed-batch cultures was 16-fold greater than attained by Kang et al. [17]. Our biomass specific yield of AAT was 0.022 g·g⁻¹, or about 8-fold greater than the best case reported.

The concentrations and productivities of the biomass and AAT in the fermenters are shown in Figs. 2 and 3, respectively. The high productivities of biomass and AAT attained in the fed-batch cultures were associated with the precisely

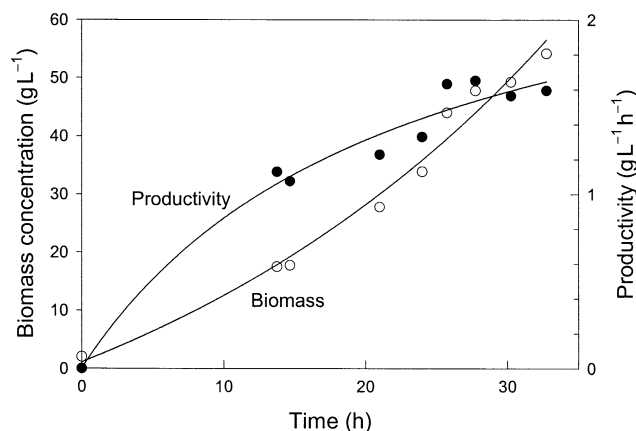


Fig. 2. Variations in biomass concentration and productivity in fed-batch fermentor.

controlled feeding which suppressed fermentative metabolism and the production of ethanol. The feeding rate was controlled by monitoring the respiratory quotient, which never exceeded 1.05 throughout the fed-batch phase. In this method, after a certain amount of feed had been added, the feeding was stopped until the dissolved oxygen (DO) concentration and the pH began to rise. A rise in DO occurred whenever consumption ceased because of exhaustion of the glucose. This method has been proved in the past for attaining high-density cultures [5,11,12]. The respiratory quotient (RQ) and the biomass concentration (X) could be correlated with the concentration of ethanol, as follows:

$$[\text{Ethanol, g}\cdot\text{L}^{-1}] = 0.3 \times 10^{0.025X} + 0.7 \times 10^{RQ}. \quad (3)$$

Equation 3 applied when the glucose concentration in the broth was maintained at sparingly low levels. The measured ethanol concentration and the concentration values calculated with Eq. 3 generally agreed quite closely (Fig. 4).

An exponential feed strategy was used, with the feed rate dependent on time t (h) as follows:

$$\text{Feeding rate} = 3.2 \times 10^{-4} t^{4.8}. \quad (4)$$

The biomass growth during fed-batch culture was satisfactorily described by the following relationship:

$$\frac{dX}{dt} = \mu X \quad (5)$$

Table 1.
Comparison of productivities in various modes of fermentation

Culture mode	Maximum concentration (g·L ⁻¹)		Maximum productivity (g·L ⁻¹ h ⁻¹)	
	Antitrypsin	Biomass	Antitrypsin	Biomass
Shake-flask	0.16 ± 0.04	9.1 ± 0.6	0.02	0.5
Batch fermenter	0.31 ± 0.05	15.1 ± 2.3	0.02	1.2
Fed-batch fermenter	1.23 ± 0.11	55.1 ± 3.5	0.04	1.6

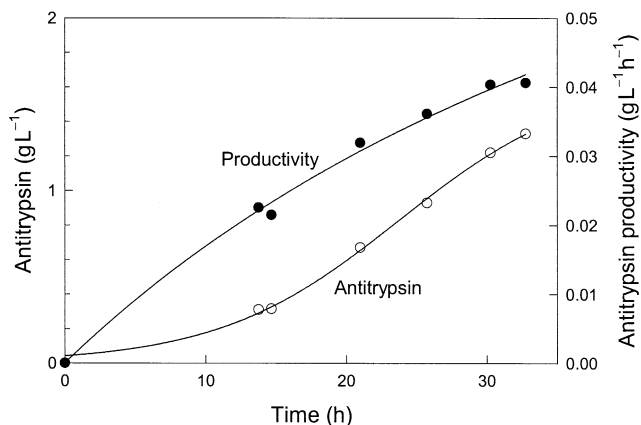


Fig. 3. Changes in AAT concentration and productivity during fed-batch culture.

where X (g) is the total biomass in the fermentor at time t (h). The specific growth rate μ had a value of 0.12 h^{-1} . The intracellular α_1 -antitrypsin production obeyed the following relationship:

$$\frac{dP}{dt} = \beta X \quad (6)$$

where the β value was $7.4 \times 10^{-4} \text{ g AAT} \cdot \text{g}^{-1} \text{ biomass} \cdot \text{h}^{-1}$. Use of these relationships allowed an estimation of the AAT produced at any time. The measured and calculated values (Eqs. 5 and 6) of the biomass and AAT are shown in Fig. 5.

Although recombinant yeasts are frequently unstable, tending to lose the transformed gene [32], the plasmid stability was not a concern in this work because the yeast used had a self selection system for vector maintenance that had been designed to function in any growth medium [16]. This was achieved by transforming a host cell that lacked an essential enzyme (triose-phosphate isomerase), with a vector that included a wildtype copy of the essential gene. The

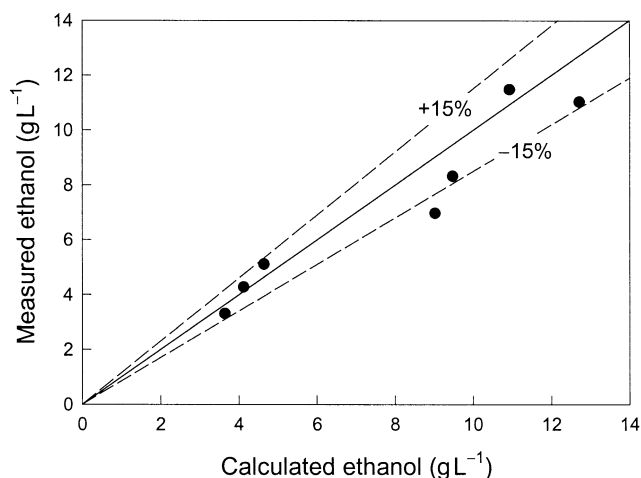


Fig. 4. Comparison of the measured ethanol concentration with values obtained using Eq. 3. The solid line represents an exact agreement.

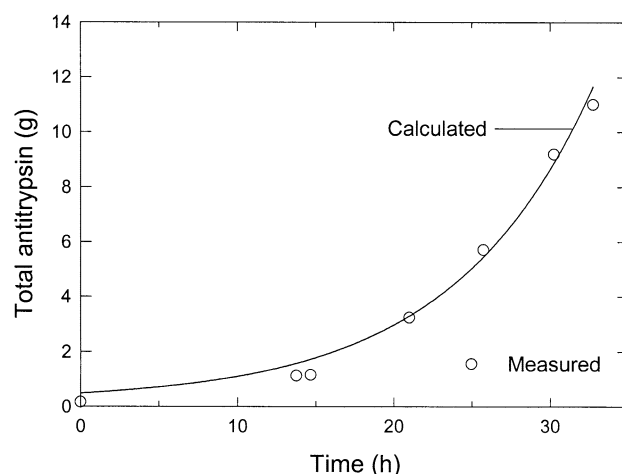
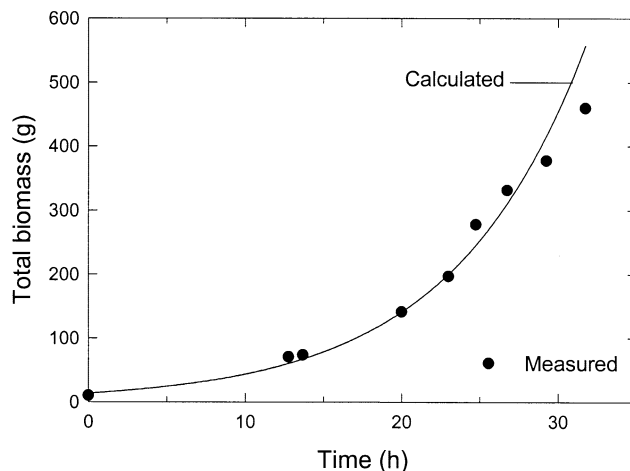


Fig. 5. Measured and calculated amounts of the total biomass and total AAT in the fermentor during culture.

cell would not survive without triose-phosphate isomerase which is necessary for central carbohydrate metabolism. Consequently, the entire biomass harvest consisted exclusively of the transformed cells.

3.2. Downstream recovery of AAT

Extracellular secreted human α_1 -antitrypsin produced by yeast has been purified from the culture medium using the following sequence of steps: ultrafiltration, ammonium sulfate fractionation (60–75% ammonium sulfate saturation), protamine sulfate treatment, and ion-exchange chromatography [19]. Similar processes have been used to recover AAT produced in bacteria such as *E. coli* [2]. The present work assessed a simple pilot scale scheme for purifying the intracellularly produced AAT. The scheme consisted of the following sequence of steps: 1. Concentration and wash of the cell slurry by dynamic microfiltration. 2. Disruption of the concentrated cell slurry in a high-speed bead mill. 3. Removal of the cell debris. 4. Ammonium sulfate fractionation of the clarified homogenate to obtain a crude prepara-

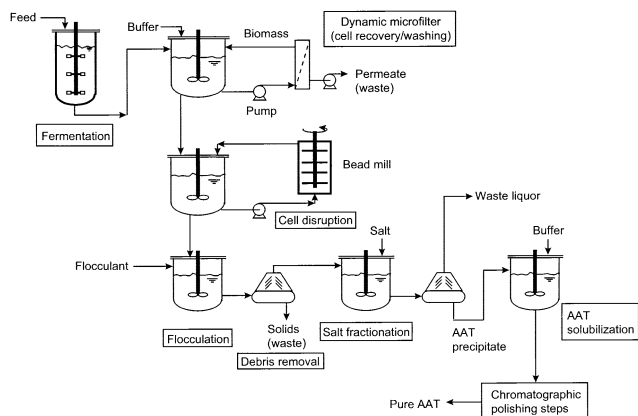


Fig. 6. Flowsheet of the recombinant AAT production and recovery process.

ration of AAT. 5. Chromatographic polishing of the AAT. A flowsheet of the recovery process is shown in Fig. 6. The recovery steps 1–4 are discussed next; chromatographic polishing is not considered here.

3.2.1. Biomass recovery

The biomass was concentrated and washed by cross-flow filtration in a dynamic filter device (see Section 2.8) that provided intense agitation at the filter surface to reduce concentration polarization. The transmembrane pressure was increased during the processing period, from an initial value of 5 psig to 11 psig. Despite an increasing transmembrane pressure, the permeate flux declined, as shown in Fig. 7. Most of the flux decline occurred during the first 10 min of processing. Obviously, concentration polarization could not be suppressed completely but a high value of the permeate flux ($\sim 90 \text{ L}\cdot\text{m}^{-2}\cdot\text{h}^{-1}$) was maintained during most of the operation. During 120 min of filtration, the biomass concentration increased four-fold to $\sim 220 \text{ g}\cdot\text{L}^{-1}$. The instantaneous biomass concentration in the retentate could be

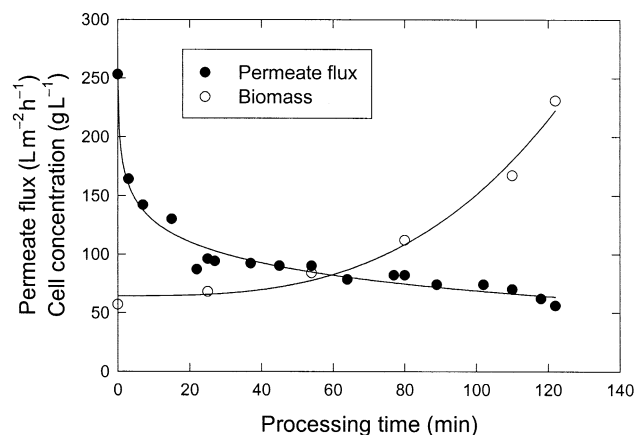


Fig. 7. Change in permeate flux and biomass concentration with processing time during dynamic microfiltration of the cell slurry.

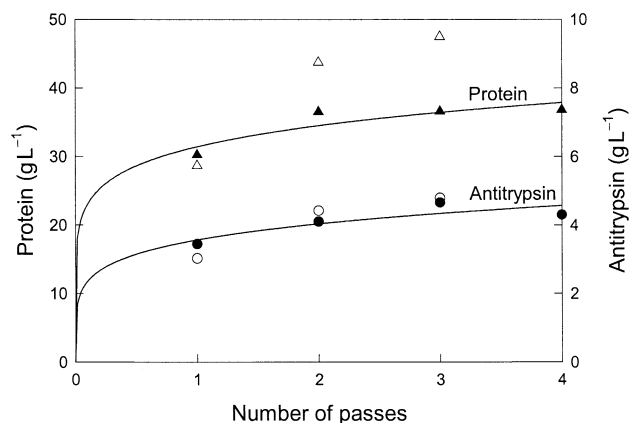


Fig. 8. Release of the total protein (triangles) and AAT (circles) during passage through the bead mill: (a) one-month-old broth (filled symbols); (b) one-week-old broth (hollow symbols).

correlated with the time of processing, using the following equation:

$$C = C_0 + 1 \times 10^{-4} t^3 \quad (7)$$

where C ($\text{g}\cdot\text{L}^{-1}$) is the concentration of the biomass after time t (s) and C_0 ($\text{g}\cdot\text{L}^{-1}$) is the initial biomass concentration in the fermentor. Equation 7 applied for a constant impeller agitation speed of 1000 rpm at the surface of the filter membrane.

3.2.2. Cell disruption and debris removal

The pattern of total protein and AAT release from cells with increasing number of passes through the bead mill, is shown in Fig. 8. Two to three passes (about 3 min total disruption time) through the bead mill were sufficient for quantitatively releasing the intracellular α_1 -antitrypsin. An excessive number of passes micronized the cell debris, making subsequent separation of solids from the homogenate difficult. The refrigerated holding time (one week or one month) of the broth prior to disruption processing had no impact on the amount of the AAT that could be released or on the ease of release of AAT (Fig. 8). This suggested that AAT was quite stable within the undisturbed cell under refrigeration (4°C). Although the amount of AAT released from cells was unaffected by storage, the amount of total protein released from one-month-old broth was significantly less than the amount released from the one-week-old cells (Fig. 8). The ratio of AAT to total released protein at complete disruption was ~ 0.08 and 0.11 for broths aged one week and one month, respectively. These values were close to $0.08 \text{ g AAT per g protein}$ reported in the cell-free filtrates of the recombinant *S. diastaticus* that secreted the produced AAT [19]. After disruption of the cells, the homogenate acquired a pH value of 5.6 – 5.8 .

Because of the concentration of the homogenate and the small dimensions of the cell debris, the homogenate could not be clarified by centrifugation at $10,000\text{-g}$ (1 h, 4°C).

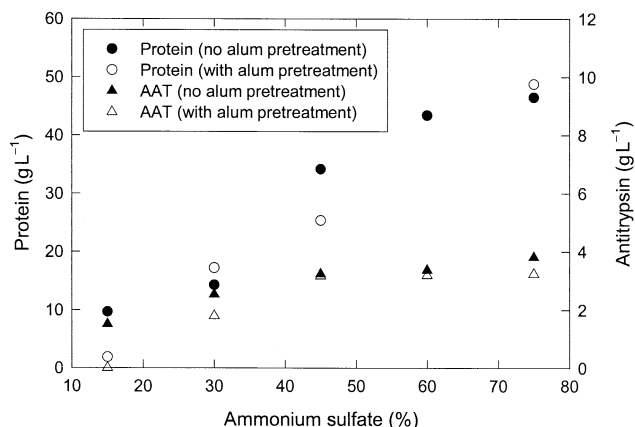


Fig. 9. Effect of aluminum chloride (alum) pretreatment on ammonium sulfate precipitation of protein and AAT from one-week-old broth. Precipitation was performed at pH 5.6.

Consequently, two types of pretreatments were evaluated that would allow centrifugal recovery of the cell debris under conditions typical of large-scale industrial centrifugation (e.g. 5,000-g, 15 min). The effective pretreatments were: (a) flocculation of the cell debris by gentle agitation with aluminum chloride [24]; and (b) dilution of the homogenate by adding two volumes of PBS (pH 7) to it. The homogenate that had been pretreated by one or both of these methods could be successfully clarified by 15 min centrifugation at 5,000-g. These pretreatments enabled centrifugal removal of solids by reducing the viscosity of the suspending fluid, by increasing the debris size, or by a combination of these factors. Potentially, microfiltration can be used for clarifying the cell homogenate. However, the dimensions of the debris and the available dynamic microfilter membrane were such that the membrane tended to clog easily and this method was not tested extensively.

3.2.3. Salt fractionation of AAT

As shown in Fig. 9, for the homogenate obtained from a one-week-old broth, pretreatment with aluminum chloride had no effect on the precipitation characteristics of protein and AAT by ammonium sulfate. This is expected because only a few drops of saturated aluminum chloride were needed for the pretreatment and the pretreatment had barely any effect on the ionic strength of the homogenate. AAT precipitated generally at > 40% of salt saturation (Fig. 9). A significantly greater concentration of ammonium sulfate was necessary for precipitating most of the AAT from the homogenate of an aged (one-month) broth (Fig. 10). This was possibly related with a lower total protein concentration in the aged broth, compared to the one-week-old broth (Fig. 9). In contrast to the present study, Kwon et al. (1995) [19] used an ammonium sulfate fractionation step with precipitation occurring at 60–75% salt saturation for recovering AAT produced in recombinant *S. diastolicus*. These differences in precipitation behavior are apparently associated

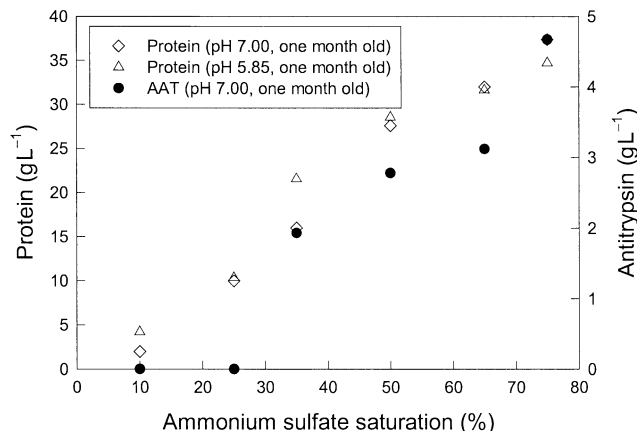


Fig. 10. Precipitation of protein and AAT by ammonium sulfate. The pH 5.85 homogenate had been pretreated with aluminum chloride.

with differences in glycosylation of the AAT produced in the two yeasts [18]. The pH value of the homogenate had barely any effect on the salt precipitation of AAT and protein over the pH range of 5.6 and 7.0. Representative data at two pH values are shown in Fig. 10.

Salt precipitation as used here provided an easy method of concentrating the AAT from a large volume of solution; however, this method does not seem satisfactory for preferentially recovering AAT from other proteins because it does not greatly affect the AAT to protein ratio in the precipitate.

3.3. Stability of AAT

Stability of the α_1 -antitrypsin has been a concern in the literature [16,27]. Compared to wild-type human α_1 -antitrypsin, certain site-specific mutagenized forms of α_1 -antitrypsin are more resistant against oxidation and other types of denaturation [16]. In view of this, and for establishing efficient processing strategies [7], an assessment of the stability of recombinant AAT in the crude clarified homogenate was undertaken. The stability of AAT in the homogenate was assessed over 9 h of incubation at 25°C and in the absence of microbial contamination. The results are shown in Fig. 11. During incubation, AAT activity declined at an average rate of $1.81 \times 10^{-3} \text{ g L}^{-1} \text{ h}^{-1}$ (zero order kinetics). Approximately 12% of the total activity was lost over 9 h. The loss was attributed to a potential presence of proteolytic enzymes in the crude homogenate. Significantly, the rate of decline in AAT activity did not depend on the stage of harvest of the broth (Fig. 11). The cells harvested at 29 h had a substantially higher AAT content than cells harvested earlier in the fermentation (Fig. 11).

3.4. Comparative assessment production schemes

The AAT production system used here produced the product intracellularly in a soluble active state. Other re-

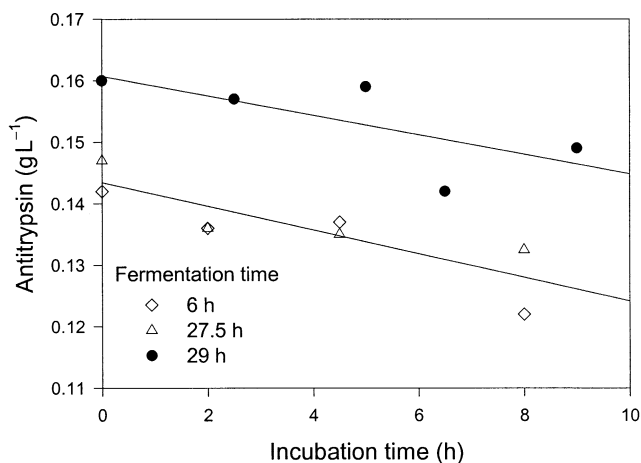


Fig. 11. Decline in antitrypsin concentration in the crude homogenates obtained at various stages of fermentation and incubated for various periods.

ported recombinant production systems are capable of secreting the active AAT [17]. Here we compare these two modes of production.

Normally, if a product is secreted then its production is a little less expensive than if the same product was produced and retained within the cell. However, this is so only if the concentrations of the product in the broths of the secretory producer and the intracellular producer are identical. In other cases, an intracellularly retained product may well be produced less expensively than the same product from a secretory system.

For our production system, the AAT concentration attained at the end of the fermentation was $1.23 \text{ g}\cdot\text{L}^{-1}$ compared to a best reported concentration of $0.075 \text{ g}\cdot\text{L}^{-1}$ for the secreted AAT [17]. Thus, our yield at the fermentation stage was 16.4-fold greater than that of the secretory production system. With this kind of difference in yields, even if we finally recovered only 70% of the product (a low value) we will be recovering 0.861 g AAT per liter of broth. In contrast, if 100% of the secreted product was recoverable, only 0.075 g of AAT will be obtained from a liter of broth. For relative comparison, we assume that the secreted product can be recovered at a nominal cost of \$1 per liter of broth and the worst case cost of recovery of the intracellular product is twice as high as that of the secreted product. Considering this, we should be able to recover the secreted product at $1/0.075$, or \$13 per g of product. The intracellular product will then be recovered at $2/0.861$, or \$2.32 per g of product. This analysis is valid because we are not concerned with the absolute cost of production of the two products but only with the relative costs of production. Clearly, because of its much greater yield the intracellular product can be produced at a cost that is less than 18% of the cost of producing the secreted product! The real cost of producing the intracellular product will be lower because the assumed relative recovery cost of \$2 is too high for this case. Incidentally, if the recovery of the intracellular product was 10-fold more

expensive than the recovery of the secreted product, our intracellular production process will still retain its cost advantage.

Neither previous work, nor our study purified the product to homogeneity but the issue of purity is not relevant to a comparison of the two processes, as discussed here. In general, there are no insurmountable technical hurdles to producing intracellular products economically on large scale and many intracellular microbial products are in commercial production [8].

4. Concluding remarks

A high cell concentration of $> 55 \text{ g}\cdot\text{L}^{-1}$ can be attained in controlled fed-batch culture of *S. cerevisiae* ATCC 20699 to provide a recombinant AAT concentration of $> 1.2 \text{ g}\cdot\text{L}^{-1}$. The AAT productivity in such fed-batch cultures is $\geq 0.04 \text{ g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ and the maximum specific growth rate of biomass is about 0.12 h^{-1} . The attainable concentration of AAT and biomass in controlled fed-batch fermentations are much greater than possible in batch cultures. A controlled environment and feeding rate tailored to minimize glucose accumulation help improve biomass yield and AAT productivity. The recombinant AAT productivity and concentration attainable with *S. cerevisiae* ATCC 20699 are much greater than have been reported for any other production system.

A suitable scheme for large-scale recovery of AAT from the biomass was proved. The scheme consisted of the following steps: 1. Four-fold concentration of the yeast biomass by dynamic microfiltration; 2. Disruption of the cells in a mechanical bead mill for a total retention period of about 3 min; 4. Removal of cell debris; and 5. Ammonium sulfate fractionation of the homogenate to recover the crude AAT. Potentially, the crude AAT can be further purified by chromatographic methods.

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