LIST OF FIGURES

Fig. 1.1 Three dimensional (3D) structure of the rhAT III (PDB ID: 4EB1) 3

Fig. 2.1 Contribution of various biopharmaceutical products in healthcare market 11

Fig. 2.2 Differential interference contrast (DIC) microscopic image of S. cerevisiae cells 14

Fig. 2.3 Various expression systems used in the production of approved therapeutic recombinant proteins 18

Fig. 2.4 The microbial growth curve with various growth phases 22

Fig. 2.5 Fed-batch fermentation profile with growth curve 23

Fig. 2.6 Galactose molecule 25

Fig. 2.7 Illustration of central composite designs with variables and Box-Behnken design with variables 28

Fig. 2.8 Pressure driven membrane processes and their separation characteristics 30

Fig. 2.9 Blood coagulation system 41

Fig. 2.10 Effects of thrombin in simplified mode 47

Fig. 2.11 Mechanism of the action of antithrombin against thrombin 49

Fig. 2.12 Antithrombin III location 50
**Fig. 4.1** The complete process flow chart for gene cloning, expression and characterization of rhAT III

**Fig. 4.2** Transformation efficiency analysis of bacterial cells: pGEM-T with AT III gene construct (5 ng) was transformed to both JM109 and TOP 10 competent cells through chemical transformation. Maximum transformation efficiency ($1 \times 10^{10}$) was observed in TOP 10 cells when compared to JM109 cells ($2 \times 10^9$)

**Fig. 4.3** Transformation efficiency analysis of pGEM-T along with AT III gene construct in Top 10 cells through chemical and electroporation transformation methods. Maximum transformation efficiency ($1.4 \times 10^{10}$) was observed through electroporation method when compared to chemical transformation ($1 \times 10^{10}$) methods

**Fig. 4.4** pGEM-T with AT III gene was isolated from overnight TOP10 culture and analyzed by 1 % agarose gel electrophoresis. Lane 1 and 2 show purified plasmid with molecular weight of approximately 4.5 kbp and compared it with molecular weight marker (Lane M)

**Fig. 4.5** Samples of the PCR products were analysed through 1.2 % agarose gel electrophoresis and stained with ethidium bromide. Lane M shows size markers of a DNA ladder, lanes 1 and 2 show the position of migration of the antithrombin product (predicted size, 1425 bp)

**Fig. 4.6** Restriction digestion analysis of clone (pYES2/CT with AT III gene): Lane 1 shows Uncut DNA/Undigested DNA (pYES2); Lane 2 shows Digests of EcoRI & XhoI; Lane M shows Marker (Supermix DNA ladder, MCLAB products)

**Fig. 4.7** Analysis of rhAT III by SDS-PAGE analyses. His-tagged rhAT III was separated from *Saccharomyces cerevisiae* BY4741 culture media by binding with Ni-NTA His bind resin. Flow through (FT), washes
(W1, W2 and W3) and elutions (E1 and E2) of rhAT III samples along with medium range molecular weight marker (Lane M) were analyzed using 12 % SDS-PAGE.

**Fig. 4.8** Two-dimensional electrophoresis analysis of rhAT III. rhAT III separation using IEF on pH 4-7 strip and followed by 12 % SDS-PAGE. After separation of rhAT III, gel was stained with ezee blue direct stainer solution.

**Fig. 4.9** Western blot analysis of rhAT III from two separate purification runs. Approximately 20 µg of purified protein was loaded onto 12 % SDS-PAGE and transferred to nitrocellulose membranes, and western blot was carried out using ab92621 antibody against rhAT III protein.

**Fig. 4.10** Hemagglutination inhibition assay of rhAT III. Well 1-10: Sheet like agglutination was observed when a rhAT III is present. Well 0 and Control (C): Button like sedimentation was observed when rhAT III is absent.

**Fig. 5.1** A detailed flow chart of integrated statistical design of experiments for medium optimization.

**Fig. 5.2** Pareto chart experimental analysis shows the effect of medium components on rhAT III yield. Where A = raffinose, B = sucrose, C = starch, D = fructose, F = glutamic acid, G = Soy peptone, J = peptone, K = Ammonium nitrate and L = vitamin mixture.

**Fig. 5.3** Residual plot analysis of rhAT III against the experimental order.

**Fig. 5.4** Predicted values against actual values of rhAT III.

**Fig. 5.5 A-C:** 3D and 2D response surface plots showing the effects of significant variables on rhAT III yield through *S. cerevisiae* BY4741.
A. Interaction effect of raffinose (g/L) and glutamic acid (g/L) \( A_1 A_2 \); 114
B. Interaction effect of raffinose (g/L) and vitamin mix (mg/L) \( A_1 A_3 \); 115
C. Interaction effect glutamic acid and vitamin mix \( A_2 A_3 \). 116

**Fig. 6.1** The complete up and down stream strategies flow sheet for rhAT III production 123

**Fig. 6.2** Batch fermentation process profile of rhAT III from *Saccharomyces cerevisiae* BY4741. The maximum cell dry weight yield (8.3 g/L) and rhAT III concentration (191 mg/L) were observed. The process variables such as rpm and rhAT III concentration values were multiplied with 10 according to their relevant axis values 125

**Fig. 6.3** Fed-batch fermentation process variables of rhAT III from *Saccharomyces cerevisiae* BY4741. Initially, the process was operated in batch mode up to 24 hours, and the fed-batch process was ended at 84 hours. The addition of limiting substrate was started at 6.8 g/L dry cell weight, and the maximum biomass yield (11.2 g/L) and rhAT III concentration (312 mg/L) were observed at 84 hours. The process variables such as rpm and rhAT III concentration values were multiplied with 10 according to their relevant axis values 126

**Fig. 6.4** Native PAGE analysis of rhAT III lysate samples with 5 and 10 mL of lysis buffer. Lane 1 and 2 shows the rhAT III test samples. Lane 3 and 4 shows control rhAT III samples. Similar antithrombin (58 kDa) bands were observed with respect to different volumes of lysis buffer control (5 and 10 mL) and the molecular weight of both purified and control rhAT III (58 kDa) was compared with molecular weight marker (lane M) 129

**Fig. 6.5** RP-HPLC profiles of cell lysate before and after cross flow filtration. The maximum purity of rhAT III (≥ 94 %) was achieved with temperature (22 °C) and transmembrane pressure (16 psi) 132
**Fig. 6.6** FPLC chromatogram of rhAT III eluted through the IEC with SP Sepharose - FF resin. Monomer peak (indicated with *) of rhAT III with a good resolution was separated with maximum yield of recovery and purity of 55 and ≥ 98 % respectively.

**Fig. 6.7** RP-HPLC analysis of standard and purified rhAT III eluted through a phenomenex C18 column (250 x 4.6 mm) column. The peaks were auto scaled according to the largest intensity peak. The maximum purity of the rhAT III (≥ 98 %) was observed with retention time 22.756 min.

**Fig. 6.8** Secondary structure analysis of rhAT III by CD spectroscopy. The CD spectra of both the standard and purified rhAT III were shown as α-helix (32 %), β-sheet (16 %), beta turns (10 %) and polypeptide chain components in the form of random coil (6 %).

**Fig. 6.9** FT-IR absorbance spectra of the purified and reference standard rhAT III. Spectra shows the amide I and II bands, which are the backbone of the protein polypeptide chain. The secondary structural components of amide I region consists of α - helix (1663 ± 2 cm⁻¹) and β - sheet (1669 ± 2 cm⁻¹).

**Fig. 6.10** SDS-PAGE analysis of rhAT III. Lane 1 shows rhAT III reference standard and lane 2 shows purified rhAT III. The molecular weight of both purified and reference rhAT III (58 kDa) was compared with molecular marker (lane M).

**Fig. 6.11** Western blot analysis of the purified rhAT III with a reference standard. Lane 1 shows the purified rhAT III. Lane 2 shows standard rhAT III. Western blot analysis of rhAT III was performed using polyclonal antibody generated from the rabbits and horseradish peroxidase (HRPO) - conjugated goat anti rabbit IgG antibody.
Fig. 6.12  LC-MS analysis of rhAT III. Molecular mass of purified rhAT III (57995.36 Da) was compared with the theoretical molecular weight of antithrombin III, which is confirmed by the Bioconfirm software

Fig. 6.13 Biological potency of rhAT III by Testzym antithrombin III 2 assay kit (Daiichi Pure Chemical, Tokyo). A comparison profile of purified and reference rhAT III activity against various rhAT III concentrations was presented. Biological activity profiles of both purified and reference rhAT III showed major correlation. All the above experiments were performed in triplicates with mean ± standard deviation (P < 0.005)

Fig. 7.1 Complete process flow sheet for rhAT III purification through different purification strategies

Fig. 7.2 Clarification and concentration of rhAT III. The rhAT III filtration performance is shown during ultra filtration with hollow fiber cartridge. rhAT III concentration was shown increasing with respective to the decrease in flux as a function of time; (■) rhAT III concentration; (♦) flux

Fig. 7.3 The effect of mobile pH on rhAT III purification. The maximum recovery yield (55 %) and purity (98 %) of rhAT III was shown with pH 8.5. Filled diamond represents recovery yield; Filled square represents purity. The experimental result analyses indicate mean values with standard variation (SV) of repetitive (duplicates) trials

Fig. 7.4 The effect of flow rate on rhAT III purification. The maximum recovery yield (66 %) and purity (99 %) of rhAT III was shown with 1 mL/min flow rate. Filled diamond represents recovery yi-
eld; *filled square* represents purity. The experimental result analyses indicate mean values with standard variation (SV) of repetitive (duplicates) trials.

**Fig. 7.5** The effect of sample volume on rhAT III purification. The maximum recovery yield (60 %) and purity (98 %) of rhAT III was achieved with 2 mL sample volume. *Filled diamond* represents recovery yield; *filled square* represents purity. The experimental result analyses indicate mean values with standard variation (SV) of repetitive (duplicates) trials.

**Fig. 7.6** The effect of protein concentration on rhAT III purification. The maximum recovery yield (67 %) and purity (99 %) of rhAT III was shown with 2 mg/mL concentration. *Filled diamond* represents recovery yield; *filled square* represents purity. The experimental result analyses indicate mean values with standard variation (SV) of repetitive (duplicates) trials.

**Fig. 7.7** Fast protein liquid chromatogram of rhAT III eluted from the size exclusion chromatographic (SEC) column. The maximum yield (67 %) and purity (99 %) of rhAT III is shown under optimum conditions: stationary phase: Superose 12; phosphate buffer with pH: 8.5; flow rate: 1mL/min; sample volume: 2 mL and sample concentration: 2 mg/mL.

**Fig. 7.8** RP-HPLC analysis of rhAT III. The purity of the rhAT III was shown to be \( \geq 98 \% \).

**Fig. 7.9** SDS-PAGE analysis of rhAT III. Lane M shows Marker; Lane 1 shows purified rhAT III; Lane 2 shows reference rhAT III. A single target band was observed with molecular weight of approximately 58 kDa and which is similar to the reference standard.

**Fig. 7.10** Western blot analysis of rhAT III. Both purified and reference
standard rhAT III bands were characterized using polyclonal antibody and HRPO conjugated goat IgG antibodies against rhAT III 158