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Full Length Research Paper

Scale-up of processes to isolate the misstargeted rBm86 protein from *Pichia pastoris*

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Negative effects of the tick *Boophilus microplus* on cattle vaccinated with the natural protein, Bm86, isolated from the tick gut epithelial cells, have been progressively reduced. Gene coding for the protein Bm86 has been cloned and expressed in the yeast *Pichia pastoris* and a technology has been developed to obtain the vaccine Gavac™ for the control of cattle tick. An unexpected misstargeting of the recombinant protein took place during fermentation process and the protein remained held into the cell strongly associated to the insoluble fractions of the yeast. In consequence, stages to isolate the target protein involved cell washing and harvesting, cell disruption, pellet-cell washing, solubilization, and ultrafiltration. Scale up of mechanical cell disruption was successfully achieved, from a laboratory bead mill to a pilot plant mill, keeping constant the total retention time. Modelling of pellet-cell washing allowed finding optimal conditions to remove 88.9 and 63% of contaminant proteins at laboratory and pilot plant scales, respectively. Diafiltration was found to be the most economic operation to remove urea from denatured protein solution. Statistical analysis of 18 production batches showed that rBm86 extraction process is under control. Here we report the scale up criteria applied in recovery steps and results are discussed regarding to each unit operation, as a contribution to the scale-up methodology for downstream processes.

Key words: Scale-up, downstream process, Bm86 protein, *Pichia pastoris,* vaccine.

INTRODUCTION

Vaccination with the rBm86 protein is a promising tool for the progressive control of the cattle tick *Boophilus microplus* (Rand et al., 1989; Willadsen et al., 1989, 1995; Tellam et al., 1992; Richardson et al., 1993; Rodriguez et al., 1995; de la Fuente et al., 1997; Boue et al., 2004). Native Bm86 is an 89 kDa membrane glycoprotein which is anchored on the extracellular surface of tick gut cells by a GPI-anchor domain (Richardson et al., 1993). The gene coding for Bm86 has been cloned and expressed in *Aspergillus niger, Aspergillus nidulans* (Turnbull et al., 1990), *Spiroqueta frugiparda* (Richardson et al., 1993), *Escherichia coli* (Rand et al., 1989) and *Pichia pastoris* (Rodriguez et al., 1995). While reports on protein expression in *Pichia pastoris* are ubiquitous in literature (Buckholz and Gleeson, 1991; Young et al., 1991; Cregg et al., 1993; Faber et al., 1995), papers dealing with large-scale isolation are not frequent.

An unexpected sorting of the recombinant protein held up rBm86 within cells, and generated a set of issues on the scale-up strategy. The sub-cellular location of rBm86, when expressed in *P. pastoris*, can be characterized by a strong affinity for membranes of the yeast, which define the initial unit operations and its sequence for the

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isolation process, without allowing any other alternative operation sequence. To our knowledge, the scale-up of a process, starting from these narrow boundaries has not been reported in the literature. Consequently, it was necessary to include a mechanical cell disruption step in downstream processing. The strong interaction between the recombinant protein and solid fractions of cell, drove us to develop a solubilization process to isolate rBm86. Large-scale isolation of rBm86 involving mixing, centrifugation and mechanical cell disruption operations has been reported elsewhere (Canales et al., 1997).

Many variables were involved in the scale-up; therefore rational criteria had to be applied to the scale-up of each unit operation. Cells washing and harvesting were scaled-up keeping constant cell concentration and relative centrifugal force. Maintaining identical design of impellers and chamber, bead loading, impeller peripheral speed and retention time was followed as scale up criterion for mechanical cell disruption. Mixing steps were scaled-up preserving equal mixing time at both scales. Pellet-cell washing was investigated searching for maximal contaminant protein removal and minimal losses of cell debris. At pilot plant scale three variants of membrane processes were tested to find a procedure to remove urea from a denatured protein solution with minimal losses. This study represents an important step towards implementing a methodology for the scale-up of isolation of recombinant proteins from insoluble fractions of yeasts. This paper can be considered as a contribution to the solution of this significant challenge.

MATERIALS AND METHODS

P. pastoris

P. pastoris was obtained from 100 L fermentations, which were performed in a fed- batch mode. Cells were cultivated in a supplemented saline medium (SSM) containing ammonium sulphate, di-ammonium hydrogen orthophosphate, di-potassium hydrogen orthophosphate, calcium chloride, vitamins and trace element. Initially, cells were grown in 3% (v/v) glycerol and upon its depletion 1% (v/v) methanol was added to induce the production of the rBm86 protein. At laboratory scale, cell washing and harvesting were performed in a floor-standing centrifuge SCR 7B (Hitachi, Tokyo, Japan) at 10 600 x g for 30 min. Cell washing and harvesting at the pilot plant scale were performed in a CSA 8 self-cleaning centrifuge (SCC) with auxiliary opening chamber (Westfalia Separator, Gelde, Germany). The technical data of pilot-plant SCC were as follows: volumetric flow up to 2000 L/h, pressure up to 6 bar, unloading time up to 16 min and relative centrifugal force (RCF) 10 600 x g.

Cell disruption

Disruptions were made in bead mills Dynomill KDL and KD5 (Willy A. Bachofen Maschinenfabrik, Basel, Switzerland) at laboratory and pilot plant scales, respectively. Cells were suspended at 400 g/L (wet weight) in a 50 mM phosphate buffer, pH 7, containing 300 mM NaCl, 1.15 mM 2-mercaptoethanol and 5 mM EDTA. Suspension was fed continuously through the grinding chamber of the mill filled with 0.50 - 0.75 mm diameter glass beads (85% of the grinding chamber volume). The technical data of the pilot-plant bead mill were as follows: peripheral speed 10 m/s, volumetric flow of cell suspension depended on experiments, heat transfer area 0.24 m^2, bead loading porosity 39%, refrigerating plant DMK30 (Willy A. Bachofen Maschinenfabrik, Basel, Switzerland), cooling capacity 2.09 kJ/s, volumetric flow of refrigerant 280 L/h, temperature up to -20°C.

Pellet-cell washing

Disrupted cells in the bead mill KDL were homogenized 10 min in each washing buffer solution by an UT-25 laboratory homogeniser (IKA, Frankfurt, Germany). The homogeneous suspension was centrifuged in a floor-standing centrifuge SCR 208 (Hitachi, Tokyo, Japan) at 10 600 x g during 30 min. Two samples of the supernatant were collected for protein and dry weight assays and the remainder was discarded. The deposit was suspended in the next buffer solution and the procedure was repeated three times up to the fourth pellet-cell washing step. First and fourth washing solutions contained 0.5 M NaCl in buffer phosphate pH 5, 7 or 9, depending on the experiment conditions. Second and third washing solutions contained 0.5 M NaCl, 1, 2 or 4 M urea in buffer phosphate pH 5, 7 or 9, according to each experiment to be performed. To investigate the influence of pH and urea concentration (Cu) on the efficiency of pellet-cell washing, the percentage of protein removal (PPR) was chosen as a response variable in each washing step, because the rBm86 protein was associated to the insoluble fraction of the yeast. The PPR was calculated as follows:

$$PPRi = \left(\frac{SPi}{TSPi}\right) \cdot 100\% \quad (1)$$

Where: SPi = soluble protein in the supernatant after pellet-cell washing step i (g), and TSPi = soluble protein fed to pellet-cell washing step i (g). The loss of cell debris (LCD) was also chosen as a response variable and calculated as:

$$LCDj = \left(\frac{DWSj}{FDWj}\right) \cdot 100\% \quad (2)$$

Where: DWSj = dry mass in the supernatant after pellet-cell washing step j (g) and FDWj = dry mass fed to pellet-cell washing step j (g), as result of multiplying volumes of feed and supernatant by corresponding dry weight concentrations. The factorial design of experiments was at 2 levels (2^2) with 2 replica and models were accepted if presented the following features: correlation coefficient > 0.9, F-ratio > F-critical, residuals randomly distributed within the range ± 2 · S (S: standard deviation) and the sum of errors was equal to zero.

Disrupted cells in the bead mill KD5 were washed suspending them in a 50 mM phosphate buffer, pH 7, containing the same salts as those used in cell disruption. Self-cleaning centrifuge described in starting material was used for phase separations at pilot plant scale. The technical data of pilot-plant mixing tanks were as follows: volume 200 L, (height/diameter) = 1, marine propeller stirrer, stirrer speed up to 500 rpm, PT120G rotor/stator homogeniser (RSH), peripheral speed 17 m/s (Kinematica AG, Lucerne, Switzerland). Mixing times up to 30 s were measured by adding 1 M NaCl to mixing tanks filled with water and recording the time to stabilise the water conductivity.

Determination of dry weight

Samples of supernatant from each washing step were centrifuged in a SCT 158 small high-speed centrifuge (Hettich Zentrifugen, Tuttlingen, Germany) at 6 710 x g during 15 min. The precipitate was suspended by vortex in a KB 10 vortex (Janke & Kunkel, Staufen, Germany) in distilled water to remove soluble fractions, centrifuged and resuspended up to 1 mL. Dry weight was determined in an MA 30 moisture analyser (Sartorius AG, Goettingen, Germany).

Solubilization of the rBm86 protein

The washed cell-debris particles were homogenized for 30 min in phosphate buffer, pH 7, containing urea or urea plus 2-mercaptoethanol. Concentrations ranging from 5 to 10 mM and of 2, 4 and 8 M were taken for 2-mercaptoethanol and urea, respectively. The solution was stirred and then a separation step was performed in the floor-standing centrifuge at 14 000 x g, for 30 min. At pilot plant scale, the washed cell- debris was suspended and strongly homogenized for 30 min by using the RSH in phosphate buffer, pH 7 containing 8 M urea, 1 mM EDTA and 5 mM 2-mercaptoethanol. After homogenization, the solution was stirred in a holding tank and then a separation step was performed in an AS 16 tube-type centrifuge (Sharples, Camberley, UK). The technical data of the pilot-plant tube-type centrifuge (TTC) were as follows: RCF 14 000 x g and volumetric flow 24 L/h.

Ultrafiltration

A DC30 tangential ultrafiltration system was used at pilot plant scale (Amicon, Massachusetts, USA). The technical data of the ultrafiltration system is the following: Polysulfone hollow fibber cartridges, filtration area 0.45 m^2, and cut-off 30 kDa. Operation parameters were the following: transmembrane pressure 1.4 bar, circulation rate 0.15 m/s. After each experiment, the system was cleaned with a 0.2 M NaOH solution for 12 h. Cleaning was verified by measuring the water flow through membranes.

Diafiltration

Diafiltration solution contained 25 mM sodiun phosphate and sodium hydroxide at pH 11.5. Diavolume was calculated by the following equation:

$$Dv = \frac{Va}{Vo} = \ln\left(\frac{Cuo}{Cuf}\right) \qquad (3)$$

Where: Va is the volume of diafiltration buffer added, Vo is the diafiltration volume, Dv is the diavolume, C_{UO} is the urea concentration of the protein solution before diafiltration, C_{Uf} is the final urea concentration after diafiltration (Kurnik et al., 1995). The following operation sequences were assayed: (i) Denatured protein solution was diafiltrated with 2.3 diavolumes during 2 h, (ii) Protein solution was diluted suddenly from 8 M to 4 M urea concentration and ultrafiltrated up to the starting volume and diafiltrated with 1.6 diavolumes, during 2 h and (iii) Protein solution was diluted from 8 M to 0.8 M urea concentration in 2 h and ultrafiltrated up to the starting volume for 2 h.

Determination of viscosity

Viscosity determinations were made at 20ºC by taking up flow curves at shear rates between 75 and 1 875 s^{-1} in a programmable rheometer (Brookfield, Loughton, UK). A cone and plate measuring system was used with a cup containing a sample volume of 500 µL. Determinations of viscosity were applied to disrupted cells and diafiltration samples.

Determination of protein concentration

Samples for protein determination were first centrifuged in a SCT 15B small high-speed centrifuge (Hettich Zentrifugen, Tuttlingen, Germany) at 6 710 x g for 15 min and supernatants were directly analysed by the Bradford's method (Bradford MM, 1976) with bovine serum albumin as standard.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electroforesis (SDS-PAGE) was performed as described by Laemmli (1970). Electrophoretic runs were performed in 10% gels with samples under standard reduction and denaturing conditions using an electrophoresis buffer containing 25 mM Tris-HCl, 192 mM glycine pH 8.4, and 0.1% SDS. Samples were first mixed with sample buffer: 4% SDS, 1.25 M 2-mercaptoethanol, 0.2% glycerol, 180 mM Tris-HCl pH 6.9, and 0.01% bromophenol blue. The slabs were stained with a solution of 0.05% Coomassie Blue G-25 in 10% acetic acid, 10% methanol solution, and distained by washing in the same solution without Coomassie Blue. Immunoblotting was not used as a routine process control method, because a competition enzyme linked immunosorbent assay allowed to identify and quantify rBm86 by a single assay.

Determination of the rBm86 protein concentration

A competition enzyme linked immunoabsorbent assay (ELISA) was used to determine the rBm86 protein concentration. Maxisorp Nunc F96 microtiterplates were coated over night at 4ºC with 100 µL per well of a 1 µg/mL solution of rBm86 in coating buffer (0.05 M Na_2CO_3, 0.05 M $NaHCO_3$, pH 9.6). After washing three times with phosphate buffer solution (PBS) containing 0.05% Tween 20 (PBST), the unbound reactive sites were blocked with 3% (w/v) skimmed milk in PBS for 1 h at 37ºC. 50 µL of the monoclonal antibody anti-rBm86 (SSBm1) in PBS were added to 50 µL of triplicates samples or standards to give a final concentration of 62.5 ng/mL and a set of standards ranging from 1 µg/mL to 88 ng/mL. After 1 h at room temperature with a gentle shaking, plates were washed three times with PBST, 100 µL of a second antibody (anti-mouse IgG labelled with horseradish peroxidase) diluted 1:6000 (v/v) in PBS was added and microtiter wells were incubated at 37ºC for 30 min. Plates were washed five times and then 100 µL of substrate solution (0.1 M Na_2HPO_4, 0.048 M $C_6H_8O_7.H_20$, pH 5.5, containing 45 mg/mL OPD and 30% (v/v) H_2O_2 was added). The reaction was stopped with 50 µL of 2.5 M H_2SO_4 after 15 min at room temperature and the absorbance was measured at 492 nm using a plate reader.

Statistic analysis

The mean value of rBm86 concentration and the standard deviation were calculated from results of 18 production batches. Upper and lower control limits (UCL and LCL) were also calculated as follows:

$$UCL = \overline{X} + 3 \cdot S \qquad (4)$$

$$LCL = \overline{X} - 3 \cdot S \qquad (5)$$

Where: $\overline{X}$ = mean value of the rBm86 protein concentration and S = standard deviation of the rBm86 protein concentration. The control graphic of elements was made to determine if the recovery process was under control.

RESULTS AND DISCUSSION

Cell concentration reached up to 250 g/L (wet weight) during 90 h of growth and presence of the protein rBm86, throughout fermentation, was never detected in the supporting medium. After 80 h fermentation, an immunochemistry study demonstrated that the recombinant protein was held up within cells, distributed in the cytosol (Canales et al., 1997, 1998) and the expression level was about 1.5 g/L (Rodriguez et al., 1994). Cell harvesting parameters such as: volumetric flow, pressure, partial deloading time, opening time, total unloading time and cell concentration were empirically optimized. Although it was not possible to obtain a mathematical model for the recovery of biomass as a function of the six independent variables, the operation was standardized by applying the method of evolutionary operation (Box, 1954, 1962; Chanmugan and Jenskins, 1963) for maximizing recovery and process consistency. This method consists of imposing small systematic variations on each independent variable, during the operation without affecting product quality. An inspection of the physical meaning of the six independent variables suggest a direct correlation between volumetric flow, cell concentration and the time to fill the settling volume within the rotor. However, the impact of partial deloading time, opening time, unloading time and pressure on the recovery of biomass and variable interactions were not evident in this unit operation. Cell harvesting trials were scaled up to pilot plant keeping both the relative centrifugal force (RCF) and the mixing time (30 s) constant between scales. Cell concentration of 125 g/L, volumetric flow of 100 L/h, partial unloading time of 4.5 min, pressure of 4.5 bar, opening time of 6 s and a total deloading at the end of the operation were chosen as optimal conditions. Maximum recovery of 95 % (w/w) and concentration of biomass of 50 % (w/v) were obtained, under these operation parameters.

After cell harvesting, biomass at 100 g/L (dry weight) was disrupted in bead mills KDL and KD5. Total disintegration of cells was achieved after 6 min of total retention time keeping identical grinding chamber geometry and design, impellers geometry and peripheral speed, bead size and loading and cell concentration as scale up criterion. Part of generated heat in KD5 (3.67 kJ/s) exceeded the cooling capacity of the refrigerating plant (2.09 kJ/s) and was removed by cell suspension (Canales et al., 1998). We interpret this result to be a combination of insufficient heat transfer area in KD5 and increase of apparent viscosity of disrupted cell suspension during disruption process, increasing 3.49 to 7.71 mPa·s, at speed gradients between 75 and 1 875 s^{-1}.

Heat transfer area of the cooling jacket was not enough to allow the absorption of the generated heat, by the refrigerant, under a flow regime less and less turbulent due to viscosity increase. Exchange area (0.24 m^2) should be at least 2-fold to fulfil heat transfer requirements, if it is assumed that the temperature difference and the global heat transfer coefficient (U) remained constant. However, U probably was not constant, since the cell suspension viscosity changed during disruption process. The following alternatives were considered to overcome the heat generation problem: (1) reducing cell concentration in the feed, (2) increasing the heat transfer area, (3) increasing the volumetric flow of refrigerant and (4) increasing the cooling capacity of the refrigerating plant. The last alternative was chosen and a refrigerating plant with a higher capacity was purchased.

Pellet-cell washing is observed to be essentially important for removal of soluble and peripheral membrane proteins and reducing the presence of contaminant proteins in the solubilization step for the isolation of rBm86. Percent of protein removal (PPR) in the first pellet-cell washing was only affected by the pH, since urea was not included in this step. At pH 5 the PPR was 37% and at pH 9 the PPR obtained was 48%. The isoelectric point of this protein mixture has been experimentally determined as 4.5 (Paez, 1993), therefore, pH 5 is unfavourable for *P. pastoris* proteins solubilization. According to the factorial design described in materials and methods, the model obtained from experimental results should contain 4 terms. A mathematical correlation was obtained from second pellet-cell washing with a correlation coefficient of 0.99 as follows:

$$PPR = 8.194 \cdot Cu + 2.539 \cdot pH - 0.784 \cdot Cu \cdot pH - 15.139 \quad (6)$$

From equation 6 could be inferred that Cu had the highest influence on proteins removal. The interaction between Cu and pH had a detrimental effect, but the pH favoured protein solubilization. In the third washing step, the correlation coefficient was 0.93 for the following equation:

$$PPR = 1.536 \cdot Cu + 0.534 \cdot pH - 0.136 \cdot Cu \cdot pH - 0.764 \quad (7)$$

In the third washing again Cu had an overwhelming effect on protein solubilization and its interaction with pH was detrimental within the boundaries of this study. A fourth washing was studied to determine effect of pH on removal of proteins from starved debris. At pH 5 protein removal was higher (1.24%) than at pH 9 (0.58%), whichsuggested a significant difference between isoelectric points of protein mixtures from first and fourth washing. It suggests the presence of cytoplasmic

Table 1. Percent of contaminant protein removal during pellet-cell washing at laboratory and pilot plant scales.

Pellet-cell washing steps	Floor-standing centrifuge	Self-cleaning centrifuge
1	79.8	43.7
2	8.0	11.6
3	0.5	2.9
4	0.0	1.2
Concentration step	**	6.0*
Total removed protein (%)	88.3	63.0
Pellet-cell recovery (%)	73.7	74.5

*Performed in a tube-type centrifuge Sharples AS16 at pilot plant scale.
**There was no concentration step at laboratory scale.

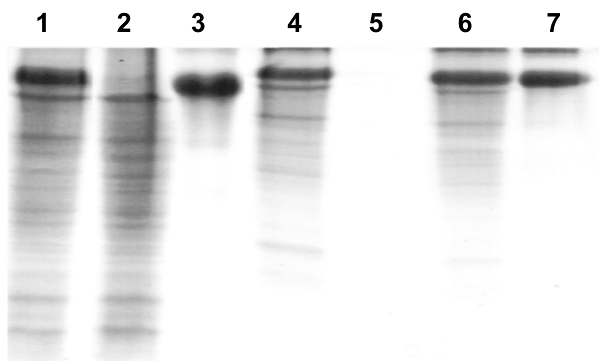

Figure 1. SDS-PAGE of rBm86 isolation steps samples. Pellet-cell of fourth washing step, lane 1; Supernatant of forth washing step, lane 2; standard solution of rBm86, lane 3; supernatant of rBm86 extraction, lane 4; pellet-cell of rBm86 extraction, lane 5; diafiltration solution containing rBm86 protein, lane 6; supernatant of acid precipitation step, lane 7.

proteins in the first washing and peripheral membrane proteins in the last one. In this study, regressions had correlation coefficients higher than 0.9 and F-ratio was always higher than F-critical. The sum of residuals was approximately equal to zero and residuals were randomly distributed within the interval ± 2 · S. Losses of debris particles at several conditions of Cu and pHs were determined. The higher loss was reached at pH 9 and Cu = 4 M, which was 2-fold the lost at pH 7 and Cu = 1 M. These results indicated that although the higher Cu improved protein removal, the loss of cell-debris was also increased at this condition, affecting target protein recovery. While washing pellets-cell, steps described in materials and methods, made possible to remove about 89% of contaminant proteins, with a pellet-cell recovery 73.7 % at the laboratory (Table 1). Alternating buffer solutions with and without 1 M urea allowed removal soluble proteins in the first pellet-cell washing under mild conditions and contaminants associated to cell debris by urea in the second and third washing. By fourth washing more contaminants were removed without including urea in the buffer solution to avoid losses of cell debris containing the rBm86 protein. Empirical optimisation of cell debris centrifugation at pilot plant scale followed the same strategy as in cell washing and harvesting, but variability of the feed from a washing step to the next one and cell debris heterogeneity impeded obtaining a recovery similar to that of the cell harvesting stage. Difference in the operating principles between the laboratory floor-standing centrifuge and the SCC influenced on unlikeness in contaminant protein removal between laboratory and pilot plant scales during pellecell washing (Table 1). Losses of pellet-cell were similar at both scales in spite of obvious differences between a floor-standing centrifuge and a SCC. The SCC removed the greatest proportion of contaminant proteins (55.3 %), during first and second washing steps, but removal was minimal from the third washing step (4.1 %). These results do not mean that last washing steps are not necessary, because non-protein contaminants were not quantified by this study. The use of a TTC could improve the contaminant protein removal, but it would reduce the productivity of this step and efficacy of a TTC remains to be demonstrated. Cell fractionation experiments of the yeast *P. pastoris* (liu et al., 1995; Waterham et al., 1996) have demonstrated the wide range of densities and particle sizes that are present in a disrupted suspension of the yeast. Therefore, achievement of optimisation of the cell debris centrifugal separation gets difficult. Several alternatives were considered to reduce the presence of cell debris particles in the supernatant and increasing the RCF of the TTC from 14 000 to 18 000 x g was chosen as the most effective. Three TTC with 18 000 x g were purchased for that purpose.

The rBm86 protein produced in *P. pastoris* appears in SDS-PAGE and immunoblotting as a wide band, similar to heterogeneous molecular species with size from 90 to 100 kDa (Rodríguez M et al, 1994). To observe rBm86 location and evaluate if there were target protein losses, pellets and supernatants from the fourth washing, protein extraction and diafiltration steps were applied in SDS-PAGE (Figure 1). Four washing steps were not enough to separate the rBm86 protein from cell debris. A strong interaction, which takes place between the target protein and the membrane system, was evident. Therefore, values of protein concentration in supernatant of washing steps can not be correlated or compared with protein concentrations in supernatant of rBm86 solubilization step.

In experiments without 2-mercaptoethanol, rBm86 extraction was not observed, even at high urea concentration. On the contrary, rBm86 solubilization in presence of 2-mercaptoethanol was highly accelerated as urea concentration rose from 2 to 8 M. More than 80% of all rBm86 associated with cell debris was extracted after 2 h when 8 M urea was used (Figure 1). Protein extraction was scaled up keeping identical mixing time (9 s in the homogenization step and 30 s in the stirring step)

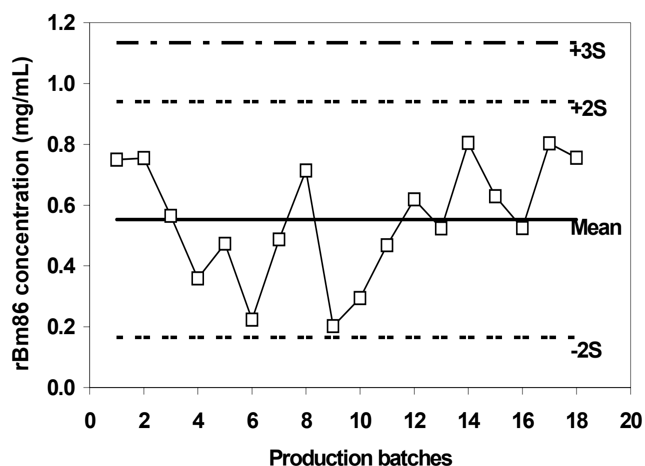

Figure 2A

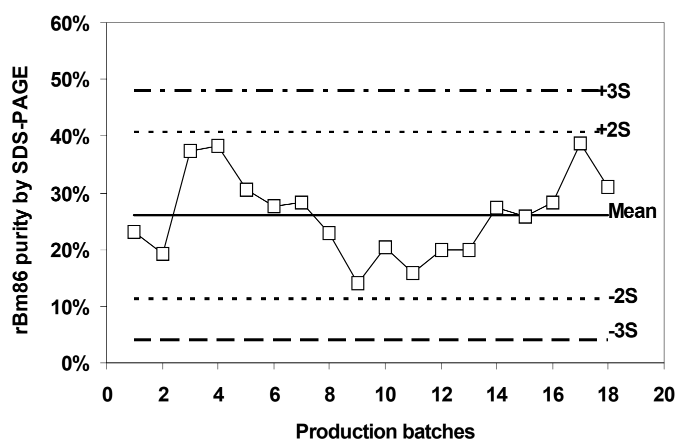

Figure 2B.

Figure 2. Control graphic of the rBm86 protein concentration (A) and purity (B) in the solubilization step of 18 production batches.

between laboratory and pilot-plant scales. The rBm86 protein was fully extracted from pellet-cell after 0.5 h homogenization and 3 h stirring at pilot plant. After centrifugation, using the TTC, rBm86 protein purity and concentration were measured. Purity of 18 production batches was 26% on average and 55% of values were higher than 26% (Figure 2A). Average rBm86 protein concentration was 0.553 g/L of supernatant and 77.7% of values were in the range from 0.4 to 0.8 g/L of supernatant (Figure 2B). It was demonstrated that the process is statistically under control (Gershon, 1991), because there were neither value out of control limits, nor three values out of the alert limit (± 2·S). There were neither seven points above or below the central straight, nor a monotony-growing decreasing trend of more than seven values was observed in the plot. However, the presence of cell debris particles in the supernatant, after centrifugal separation, was persistent. Diafiltration was the most suitable procedure to remove urea from denatured protein solution, since permeate flow kept constant. The increasing of the hydraulic resistance of the membrane due to fouling was compensated by apparent viscosity decreasing from 1.52 to 0.97 mPa · s, and pH increase from 7 to 11 during diafiltration. Similar results were obtained when applied Dilution-Concentration-Diafiltration method (D-C-DF). In concentration step permeate flow decreased 30% and kept constant in diafiltration process. Diafiltration and Dilution-Concentration-Diafiltration method showed a similar behaviour in all process parameters, but Diafiltration had some advantages compared with Dilution-Concentration method were necessary 3.9-fold less buffer volume, 1.9-fold less membrane area, 1.5-fold less working area and 2-fold less operation time by using diafiltration procedure.

The final purification of the rBm86 protein is reached with a selective acid precipitation step of the contaminant. The most of contaminant proteins are removed and a solution of rBm86 with more than 95% purity and 95% recovery is obtained in this step are obtained (Boué et al., 1997).

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