Protein Expression and Purification 123 (2016) 112–121

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Production in stirred-tank bioreactor of recombinant bovine chymosin B by a high-level expression transformant clone of *Pichia pastoris*

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ARTICLE INFO

Article history:
Received 17 November 2015
Received in revised form
21 March 2016
Accepted 22 March 2016
Available online 28 March 2016

Keywords:
Bovine chymosin
Pichia pastoris
Methanol-inducible *AOX1* promoter
Biodiesel-derived crude glycerol
Fed-batch fermentation
Protein purification
Reiterative methanol-inductions

ABSTRACT

An intense screening of *Pichia pastoris* clones transformed with the gene of bovine chymosin under methanol-inducible *AOX1* promoter was performed, obtaining a transformant clone with a higher milk-clotting activity value in comparison with our previous studies. The scaling of recombinant-chymosin production was carried out by a fed-batch strategy in a stirred-tank bioreactor using biodiesel-byproduct crude glycerol as the carbon source and pure methanol for the induction of chymosin expression, achieving a biomass concentration of 158 g DCW/L and a maximum coagulant activity of 192 IMCU/ml after 120 h of methanol induction. Recombinant bovine chymosin was purified from bioreactor-fermentation culture by a procedure including anion-exchange chromatography which allowed obtaining heterologous chymosin with high level of purity and activity; suggesting that this downstream step could be scaled up in a successful manner for chymosin purification. Thermoestability assay permitted to establish that unformulated recombinant chymosin could be stored at 5 °C without decrease of enzyme activity throughout at least 120 days. Finally, reiterative methanol-inductions of recombinant chymosin expression in bioreactor demonstrated that the reutilization of cell biomass overcame the low enzyme productivity usually reached by *P. pastoris* system.

1. Introduction

The bovine chymosin (EC3.4.23.4) is one of the main enzymes in food industry since is used for the elaboration of cheese. This enzyme is an aspartic acid protease with high milk-clotting activity and is synthesized by the cells of the abomasum mucosa of newborn calves [1]. The bovine chymosin (323 amino acids, 35.65 kDa) is expressed as a prepro-enzyme (381 amino acids, 42.18 kDa), from which a pre-sequence of 16 amino acids is cleaved in the endoplasmic reticulum when the protein is secreted. Under the acidic conditions of the gut lumen the secreted zymogene, known as prochymosin (365 amino acids, 40.48 kDa) is converted into the mature form by the autocatalytic cleavage of the 42-amino acid N-terminal prosequence [2,3]. This enzyme contains two aspartic acid residues at the active site, Asp32 and Asp215 that catalyze the selective breakdown of Phe105-Met106 peptide bond in κ-caseins which stabilize milk micelles. This specific cleavage generates the destabilization of the micelles and subsequently induces milk clotting [4]. The production of recombinant bovine chymosin in microbial expression systems, such as filamentous fungi and yeast has numerous benefits compared to the procedure of extracting authentic bovine chymosin, such as obtaining a homogeneous product due to a standardized bioprocess [5–8]. The methylotrophic yeast *Pichia (Komagataella) pastoris* has become a remarkable expression host for the synthesis of different active heterologous proteins [9–11]. The advantages of this expression organism comprise its growth to high cellular concentration on defined minimal medium [9,12], high-yield production of recombinant proteins [12,13,14], characteristic eukaryotic post-

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http://dx.doi.org/10.1016/j.pep.2016.03.008
1046-5928/

translational modifications, such as proteolytic processing, glycosylation, folding and disulfide bond formation [10,15], and the effective secretion of proteins [16]. Furthermore, *Pichia pastoris* possess the efficient methanol-inducible promoter of the alcohol oxidase I gene (*AOX1*) which is generally used to control the expression of foreign genes [17]. A distinctive feature of such promoter is that it is intensely repressed by glucose and glycerol, but induced over 1000-fold with methanol as unique carbon source, ensuring high biomass at the start of protein expression induction [9,15]. Additionally, the preference of this yeast for respiratory rather than fermentative metabolism, even at high-biomass level, prevents the accumulation of unfavorable secondary metabolites as acetic acid and ethanol [15]. Other reasons for the great success of this expression system are that *P. pastoris* is recognized as a GRAS organism [18,19] and that it is useful for scaling up heterologous protein expression in bioreactors [20]. Various chymosins from mammalian sources have been efficiently expressed using *P. pastoris* strains, such as buffalo [21], goat [22] and bovine [23,24]. In particular, bovine chymosin has been constitutive expressed in such host under the control of glyceraldehyde-3-phosphate dehydrogenase gene (*GAP*) promoter [25]. However, studies have indicated that constitutive expression of recombinant proteins could generate cytotoxic effects in *P. pastoris* [9,11].

In a previous work, we expressed bovine prochymosin B gene in GS115 *P. pastoris* strain using the pPIC9K vector under the control of *AOX1* promoter. Furthermore, we performed the scaling up of recombinant chymosin production in bioreactor and its purification by high performance gel filtration chromatography [23]. In a subsequent work, biodiesel-derived crude glycerol was used as carbon and energy source reducing the bioprocess cost. Moreover, the optimal specific growth rate (μ) during chymosin production phase was determined and activity parameters (pH and temperature) of the enzyme were characterized [24].

In this work, we carried out a screening of *P. pastoris* clones transformed with bovine prochymosin B gene in order to find clones with higher expression levels of recombinant chymosin and therefore greater milk-clotting activity, in comparison with our previous results. Furthermore, the production of recombinant bovine chymosin by a high-producer clone was scaled up in a stirred-tank bioreactor using fed-batch methanol feeding under optimized conditions. In addition, we purified the recombinant bovine chymosin by anion-exchange chromatography and evaluated the thermostability throughout storage time. Moreover, we explored the advantages of reiterative methanol inductions of chymosin expression in bioreactor.

2. Materials and methods

2.1. Strains and reagents

Clones of *P. pastoris* GS115 transformed with bovine prochymosin B gene were utilized in this study. These clones were obtained by a two-step procedure described in our previous work [23], which consisted of a histidine prototrophy selection and a subsequent geneticin resistance selection. Colonies exhibiting enhanced growth in YPD-agar medium supplemented with geneticin were selected for screening the transformed clones. Commercial recombinant bovine chymosin (Maxiren-DSM; Heerlen, Netherlands) was employed as the chymosin standard for milk clotting, SDS-PAGE and purification assays. Commercial powdered skimmed milk (Nestle; Vevey, Switzerland) was utilized as the enzymatic substrate for milk-clotting determination.

2.2. Culture media composition

P. pastoris growth on solid medium was conducted at 30 ± 1 °C employing YPD medium with (in grams per liter): peptone, 20; yeast extract, 10; glucose, 20 and agar, 20. Basal salts medium (BSM) supplemented with trace metal solution (PTM1) and biotin, based on a previously described composition [23], was used for growing *P. pastoris* clones in liquid cultures. Glucose, analytical glycerol, biodiesel-derived crude glycerol and methanol were utilized as carbon sources depending the experiment. Crude glycerol, the main byproduct of biodiesel industry, was pretreated in accordance to Chi et al. [26]. Therefore, the pH of the crude glycerol was adjusted to 6.0 with HCl to convert the soluble soaps into insoluble free-fatty acids which were precipitated. The precipitate was separated from the crude glycerol by centrifugation at 3600 *g* for 20 min. Consequently; the content of glycerol was 88% v/v and 94% v/v before and after such treatment, respectively.

2.3. Biomass and glycerol quantification

Optical density of culture samples was measured at 600 nm using an UV–Vis spectrophotometer and converted to dry cell weights (DCW, in g/L) with a previously calculated calibration curve in accordance to the formula: $OD_{600nm} = 2.337 \times DCW$, $R^2 = 0.991$. Glycerol was quantified in the cell-free supernatant samples from different culture times by the microplate-adapted periodate technique as descripted by Bondioli and Della Bella [27].

2.4. Milk-clotting analysis

Milk-clotting activity was evaluated based on the end-point dilution method [28,29]. Powdered skimmed milk was reconstituted at 26% (w/v) in 0.5 g/L $CaCl_2$ (pH 6.5), mixed at 25 °C for 30 min and preincubated at 37 °C for 20 min. Culture supernatants were serial diluted to half using 0.12 M $CH_3COOH/CH_3COONa \cdot 3H_2O$ buffer (pH 5.5) in a 96-well plastic plate. Milk (50 µl) was loaded to the well which contained the supernatant dilutions (50 µl). After stirring the mixtures, the plate was incubated at 37 °C for 10 min and centrifuged at 2000 *g* for 5 min. Milk-clotting activity was determined using the highest dilution that caused milk coagulation, visualized as the formation of clots in the bottom of the wells. Highest dilution values were compared with those achieved with commercial recombinant bovine chymosin (600 IMCU/ml) to obtain the international milk clotting units per milliliter (IMCU/ml). Milk-clotting activity of each culture sample was calculated in duplicate. As negative control, blank culture medium was used instead of the culture supernatant.

2.5. SDS-PAGE analysis

SDS-PAGE electrophoresis was performed according to Laemmli protocol [30] using 12% separating gel. Cell-free culture supernatants were mixed with cracking buffer containing SDS and β-mercaptoethanol, and heated at 100 °C for 5 min. A volume of 20 µl of cracked sample was applied to each gel lane. Electrophoresis was conducted at 180 V and 4 °C during 1 h. The gels were stained with Coomassie brilliant blue (CBB) G-250 (Sigma-Aldrich; St. Louis, MO) to visualize proteins. Standard protein markers were utilized for the estimation of molecular weight of the culture supernatant proteins. Recombinant bovine chymosin concentration in supernatants and chromatography fractions was estimated by a calibration curve of BSA standard evaluated through SDS-PAGE and band densitometry using ImageJ software (http://rsb.info.nih.gov/ij).

2.6. Screening for P. pastoris clones with high milk-clotting activity in nutrient-rich medium

In order to achieve a higher production level of recombinant bovine chymosin in comparison with previous results, we carried out a screening of *P. pastoris* clones transformed with bovine prochymosin B gene. For this, 120 transformed clones that exhibited an enhanced growth in YPD-agar medium with geneticin were utilized to inoculate YPD media contained in polypropylene deep-well plates (96 round wells, 1 ml/well, conical bottom). The deep-well plates were incubated on a rotary shaker at 300 rpm and 30 ± 1 °C. After 24 h of cultivation, induction of recombinant bovine chymosin expression was initiated by adding pure methanol up to a concentration of 1% (v/v) to the wells every 24 h of incubation for a period of 96 h. Later, the deep-well plates were incubated on shaker at 300 rpm and 25 ± 1 °C throughout methanol-induction phase as was established by Noseda et al. [23]. After induction period, plates were centrifuged at 3500 *g* for 20 min at 5 °C, and culture supernatants were utilized for milk-clotting activity determination.

2.7. Screening for P. pastoris clones with high expression level of chymosin in defined medium

Transformed clones of *P. pastoris* that presented high milk-clotting activity in YPD cultures were used to inoculate 20 ml BSM (supplemented with 4 ml/L PTM1 and 30 μg/L biotin) containing analytical glycerol (10 g/L) in 100 ml Erlenmeyer flasks. The flasks were incubated on a rotary shaker at 250 rpm and 30 ± 1 °C. After 24 h of cultivation, induction of recombinant bovine chymosin production was started by adding pure methanol to the cultures up to a concentration of 1% (v/v). The incubation was continued on shaker at 250 rpm and 25 ± 1 °C, with the addition of methanol up to a concentration of 1% (v/v) every 24 h for a period of 144 h to sustain the induction conditions as was established previously [23]. Cultures were sampled every 24 h during the methanol-induction phase for biomass measurement. Subsequently, culture samples were centrifuged at 3500 *g*, 5 °C, for 20 min and the free-cell supernatants were used in the milk-clotting analysis and SDS-PAGE electrophoresis.

2.8. Scaling up of recombinant chymosin production by a stirred-tank bioreactor

In order to perform the scaling of the production of recombinant bovine chymosin in bioreactor, fermentations were carried out by a process with four steps in accordance with Celik et al. [31], with slight modifications. Pre-treated biodiesel-derived crude glycerol was employed as the carbon and energy source of the culture medium, replacing analytical glycerol in cell growth as was reported in our previous work [24]. The first phase consisted in a batch culture using BSM medium with 40 g/L crude glycerol and supplemented with 4 ml/L PTM1 and 30 μg/L biotin, where cells achieved high biomass level while repressing foreign gene expression as the *AOX1* promoter is repressed by excess quantities of glycerol. In the second stage, crude glycerol (600 g/L solution supplemented with 12 ml/L PTM1 and 90 μg/L biotin) was fed to the culture at a growth-limiting rate to increase yeast concentration and allow the slow derepression of *AOX1* promoter [32]. Crude glycerol feeding was conducted by a fed-batch strategy, established according to the constant flow rate equation: $F = \mu_o V_o C_{Xo}/Y_{x/s}\ C_S$, where μ_o is the initial specific growth rate (h^{-1}); V_o, the initial volume (l); C_{Xo}, the initial cell concentration (g/L); $Y_{x/s}$, the cellular yield coefficient based on substrate consumption (g cell/g substrate); and C_S, the feed substrate concentration (g/L). These pre-fixed parameters were taken as: $\mu_o = 0.17\ h^{-1}$, $V_o = 3.2$ L, $Y_{x/s} = 0.60\ g_x/g_s$ and $C_S = 600$ g/L. Then, a short transition stage (6 h) was conducted by a constant feed with crude glycerol:methanol (3:1) mixture, which allows adaptation of the cells to growth in methanol. Finally, the induction phase was performed by adding pure methanol (supplemented with 12 ml/L PTM1 and 90 μg/L biotin) by a fed-batch mode with a growth-limiting rate. Such feeding strategy was given by the constant flow rate *F*, where the pre-fixed parameters were taken as: $\mu_o = 0.03\ h^{-1}$, $V_o = 4$ L, $Y_{x/s} = 0.32\ g_x/g_s$ and $C_S = 800$ g/L. The recombinant bovine prochymosin was synthesized and secreted in this period of the bioprocess. To obtain the inoculum for bioreactor fermentation, *P. pastoris* cells of a transformed clone grown on YPD agar were inoculated into a 100-ml Erlenmeyer flask containing 20 ml YPD medium and incubated overnight at 30 ± 1 °C. A volume of 200 ml of BSM with 40 g/L glycerol (supplemented with 4 ml/L PTM1 and 30 μg/L biotin) contained in a 1-L flask was inoculated with the overnight culture and incubated at 30 ± 1 °C until the culture achieved an OD_{600} of ~25. This culture was used to inoculate 3 L of supplemented BSM contained in a 6-L BioFlo 110 bioreactor (New Brunswick Scientific; Edison, NJ), which was interfaced with Biocommand Bioprocessing software (New Brunswick Scientific) for parameter control and data acquisition. Temperature was hold at 30 ± 1 °C throughout batch, glycerol fed-batch and transition periods and at 25 ± 1 °C during methanol-induction phase as was determined by Noseda et al. [23]. A pH of 5 was maintained through the first three stages, and a pH of 4 was kept in the last phase, as was established by Noseda et al. [23]; these values were automatically controlled by the addition of H_3PO_4 (85% w/w) and NH_4OH (25% v/v). The dissolved oxygen concentration (DO) was hold above 30% of saturation and controlled by agitation cascade (800–1200 rpm) and filter-sterilized air supply. The pH and dissolved oxygen concentration were monitored online with a pH electrode (Mettler-Toledo GmbH, Germany) and a polarographic probe (InPro6110/320, Mettler-Toledo GmbH), respectively. Foam formation was avoided through the addition of 0.3% (v/v) antifoam 289 (Sigma-Aldrich; St. Louis, MO). Fermentation cultures were sampled during methanol-induction phase to evaluate biomass level evolution, milk-clotting activity and protein profile by SDS-PAGE.

2.9. Purification of recombinant bovine chymosin by ionic exchange chromatography

A fermentation culture sample corresponding to a 120 h methanol fed-batch conducted with a constant flow rate was centrifuged at 3500 *g* for 15 min at 4 °C, and the supernatant was employed as recombinant chymosin source. A volume of 2 L of cell-free supernatant was subjected to microfiltration in order to remove rest of biomass and cellular debris. For this, a polysulfone-membrane hollow fiber cartridge (0.1 μm pore size, 31.8 cm L, 1.0 mm ID fibers, GE Healthcare Life Science) and a tangential flow filtration system (MPH System) was employed; this step was defined as clarification. A volume of 5 ml of the clarified supernatant was subjected to fast ultrafiltration in order to concentrate the recombinant bovine chymosin. For this, an Amicon Ultra-4 device (Millipore Corp., Billerica, MA) with a molecular weight cutoff (MWCO) of 3 kDa was used at 7500 *g* for 40 min and 5 °C. Later, 800 μl of the concentrated supernatant was injected to a Tricorn 10/50 column (GE Healthcare Life Science) packed with DEAE-Sepharose (GE) and connected to a FPLC Akta Purifier equipment (Amersham Pharmacia Biotech Inc.) to perform anion-exchange chromatography at an analytical scale. The column was equilibrated with two column volumes of buffer A which consisted of 50 mM NaH_2PO_4 at pH 7. After being washed with the same buffer, a second wash with higher ionic force was carried out with the addition of 14% of buffer B consisting of 50 mM NaH_2PO_4 and 2 M NaCl at pH 7. Proteins remaining bound were eluted with the following steps: 20%, 50%, and 100% of buffer B at 0.5 ml/min.

Representative chromatography fractions were collected and analyzed by 12% SDS-PAGE electrophoresis. Purity of chymosin was estimated by densitometry of gel image by ImageJ software.

2.10. Thermostability determination of recombinant bovine chymosin

The stability of recombinant bovine chymosin was evaluated under different temperatures during storage time. For this purpose, recombinant bovine chymosin that was purified by anionic-exchange chromatography and with an initial milk-clotting activity of 48 IMCU/ml, was stored at different temperatures (5 °C, 20 °C and 37 °C) for 120 days. Periodically, recombinant bovine chymosin was sampled and milk-clotting activity was analyzed for each storage temperature by the end-point dilution assay. The effect of temperature on recombinant bovine chymosin stability was tested in triplicate for each temperature value.

2.11. Reutilization of biomass by reiterative induction of recombinant chymosin expression in bioreactor

Reiterative methanol-induction of recombinant bovine chymosin expression was carried out from bioreactor fermentation cultures of a transformed *P. pastoris* clone in order to evaluate the reutilization of cell biomass. Hence, after a complete fermentation process performed by the procedure described above, the fermentation culture was centrifuged at 4 °C and 2500 *g* for 15 min to separate yeast cells. The supernatant was destined for recombinant chymosin purification and the cell pellet was reutilized for a subsequent fermentation process. For this, the pellet was resuspended in fresh basal salts medium supplemented with 4 ml/L PTM1 and 30 μg/L biotin. Afterwards, the bioprocess was reinitiated in the methanol-induction period by adding pure methanol (supplemented with 12 ml/L PTM1 and 90 μg/L biotin) by means of a fed-batch mode with a growth-limiting rate as detailed previously. Culture samples were withdrawn in each successive induction phase for cell concentration and milk-clotting activity determination.

3. Results

3.1. Pichia pastoris transformant clones with high level expression of recombinant chymosin

A selection of clones of *P. pastoris* transformed with the gene of bovine chymosin was carried out with the aim of finding recombinant clones with higher levels of milk-clotting activity compared with results obtained in our previous works [23,24]. Therefore, a first screening was performed by growing 120 transformed clones in rich medium with glucose as carbon source (YPD medium), from which we obtained 15 clones that achieved a milk-clotting activity 8 times higher than those with the lowest value after 96 h of methanol induction (Fig. 1). This screening allowed discarding a large amount of recombinant clones that exhibited low clotting-activity and select clones with high activity for a second analysis. Therefore, in the subsequent screening which was done by growing the selected clones in basal salt medium with glycerol as carbon source, we detected a transformant clone with the highest expression level of recombinant chymosin (Fig. 2). After 144 h of methanol induction, such transformed clone showed a milk-clotting activity 8 fold greater than the lowest value recorded. Furthermore, it is noteworthy that no differences in growth rate were observed among all recombinant clones tested in the basal salt medium (data not shown). Is important to mention that other clones exhibited a clotting activity level 4 times higher than the lowest value. The difference in clotting activity obtained from cultures in YPD and MBS media was because the growth of the transformant clones in YPD medium was higher. The chymosin-transformed clone with the highest milk-clotting activity (Clone 75, Fig. 2) was chosen to conduct the scaling up of recombinant chymosin production in a stirred-tank bioreactor by a fed-batch strategy.

3.2. Analysis of the secreted protein profile of chymosin-transformed clones

Culture supernatants corresponding to 8 transformant clones with high level expression of chymosin after 144 h of methanol induction in BSM were evaluated by SDS-PAGE to analyze its protein profile. As a result of this study it was possible to confirm that the recombinant clone of *P. pastoris* which presented the highest milk-clotting activity exhibited the maximum expression level of heterologous bovine chymosin (Clone 75, Fig. 3). Moreover, the electrophoresis gel also showed that the amount of other proteins secreted by such clone increased after 144 h of induction; however recombinant chymosin was the most abundant secreted protein representing 60% of the total secreted proteins. This is an important advantage to achieve a subsequent successful purification of the recombinant bovine chymosin.

3.3. Sustainable production of recombinant chymosin in stirred-tank bioreactor

The selected transformed clone of *P. pastoris* (Clone 75) was grown in a 6-L stirred-tank bioreactor applying a procedure that included the four phases described above. In the batch stage with biodiesel-byproduct crude glycerol as carbon source, biomass reached a maximum level of 27 g DCW/L after 24 h of fermentation. At this period, the recombinant clone presented a maximum specific growth rate (μ_{max}) of 0.16 h^{-1} and a biomass yield coefficient ($Y_{x/s}$) of 0.61 g DCW/g of glycerol. After a spike of dissolved oxygen, glycerol fed-batch step was initiated with a constant flow rate of 38 ml/h consisting of 600 g/L crude glycerol. This feeding rate was kept for 20 h, when cell concentration achieved a value of 102 g DCW/L. During this period, the transformed clone exhibited an average specific growth rate (μ) of 0.083 h^{-1} and an $Y_{x/s}$ of 0.57 g_x/g_s. After glycerol fed-batch was completed, a transition stage was carried out by feeding the culture with glycerol:methanol mixture for 8 h to permit yeast adaptation for the efficient use of methanol. At the end of this stage the biomass level reached a value of 119 g DCW/L. Subsequently, pure methanol fed-batch was started with a constant flow rate of 56 ml/h to induce the expression of recombinant bovine chymosin. Methanol induction conducted with this feeding strategy achieved a biomass accumulation of 158 g DCW/L after 120 h. In addition, during induction period the cell culture grew with an average specific growth rate (μ) of 0.024 h^{-1} and reached a cellular yield coefficient ($Y_{x/s}$) of 0.27 g_x/g_s. These values did not present significant difference with those obtained from another chymosin-transformed clone analyzed in our previous work [23]. Noteworthy, the growth-limiting feeding rate applied during the induction phase allowed the maintenance of a low residual methanol concentration in the fermentation cultures, ensuring a slower protein synthesis rate and avoiding alterations in the folding and exportation machinery [10,15]. Is important to mention that, the decrease of the specific growth rate during fermentation phases resulted from the changes generated in the fermentation mode (batch or fed-batch) and the carbon source (glycerol or methanol). The recombinant chymosin production during methanol fed-batch stage increased during this phase and reached a maximum milk-clotting activity of 192 IMCU/ml after

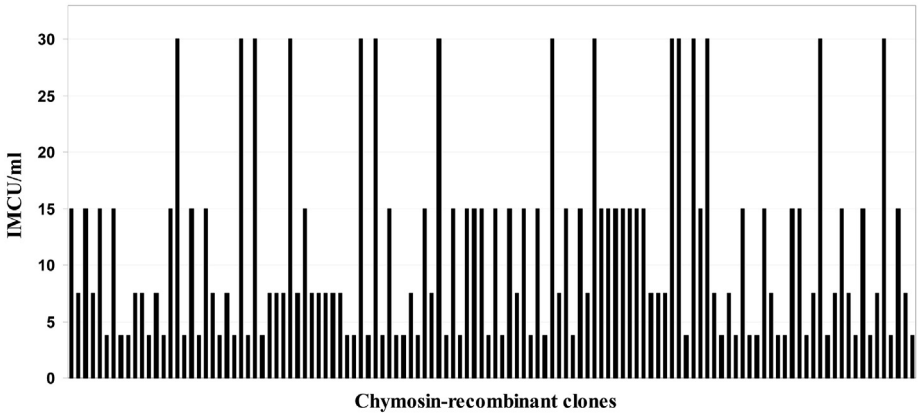

Fig. 1. Screening for *P. pastoris* clones with high milk-clotting activity in nutrient-rich medium. A total of 120 chymosin-transformed clones of *P. pastoris* were grown in YPD medium contained in deep-well plates and induced every 24 h with pure methanol during a period of 96 h. A number of 15 transformant clones exhibited a milk-clotting activity 8 times higher than those with the lowest value after 96 h of methanol induction.

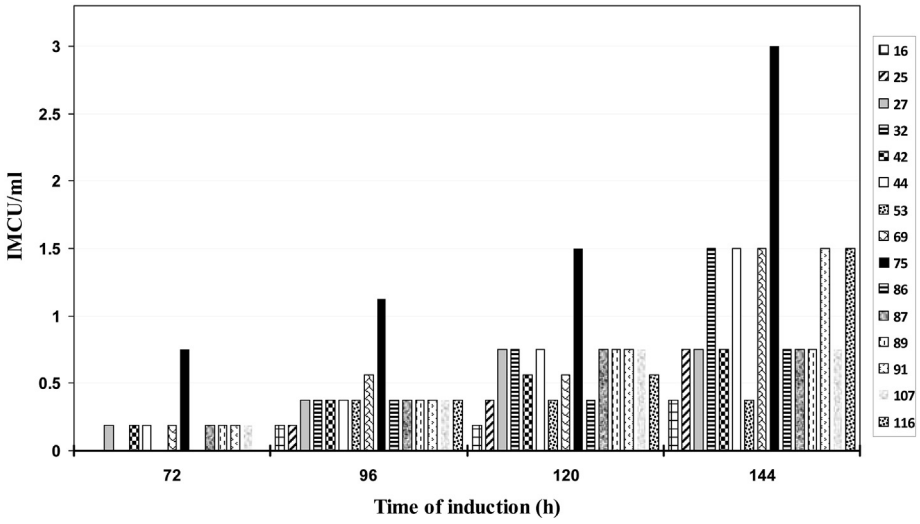

Fig. 2. Screening for *P. pastoris* clones with high milk-clotting activity in defined medium. A total of 15 chymosin-transformed clones of *P. pastoris* that presented high milk-clotting activity in YPD medium were grown in basal saline medium (BSM) contained in Erlenmeyer flasks and induced every 24 h with pure methanol during 144 h. A transformant clone (clone 75, black bar) with the highest expression level of recombinant bovine chymosin was detected from 72 h of methanol induction. After 144 h of induction, such transformed clone exhibited a milk-clotting activity 8-fold higher than the lowest value.

96 h of induction (Fig. 4). This activity value was 4-fold higher compared with that obtained in our previous work where another chymosin-transformed clone was employed [23]. After 120 h of methanol induction, no further increase in cell density and milk-clotting activity was observed with such induction strategy (Fig. 4). The use of this clone allowed to reach a coagulant activity 2-fold higher compared to fermentations in which sorbitol and ascorbic acid were added, as we indicated in our previous study [23].

3.4. SDS-PAGE analysis of recombinant chymosin produced by bioreactor fermentation

Fermentation supernatant samples from *P. pastoris* clone 75 corresponding to different methanol-induction times were analyzed by SDS-PAGE electrophoresis (Fig. 5). The samples presented two bands of recombinant bovine chymosin which is consistent with the results obtained by SDS-PAGE and western blotting from our previous works [23,24]. The recombinant chymosin bands migrated in the range of 30 and 45 kDa, as was expected due the molecular weight of bovine chymosin is 35.6 kDa [1]. The electrophoresis gel exhibited a pronounced increase in the amount of recombinant chymosin produced throughout the methanol-induction phase. This is in agreement with the results obtained from milk-clotting activity determinations presented previously. Furthermore, this analysis showed that at the start of methanol-induction period others proteins with higher molecular weights than bovine chymosin were produced. The quantity of such proteins partially decreased during the induction phase, which resulted convenient for the subsequent purification process of the recombinant bovine chymosin. The concentration of the total recombinant chymosin determined by densitometry analysis was 84 mg/L after 120 h of methanol induction. Additionally, the marked increase in the intensity of the recombinant chymosin bands confirmed that an efficient expression and secretion was achieved throughout methanol fed-batch phase.

3.5. Purification process of recombinant bovine chymosin

After initial steps of purification consisting of centrifugation and

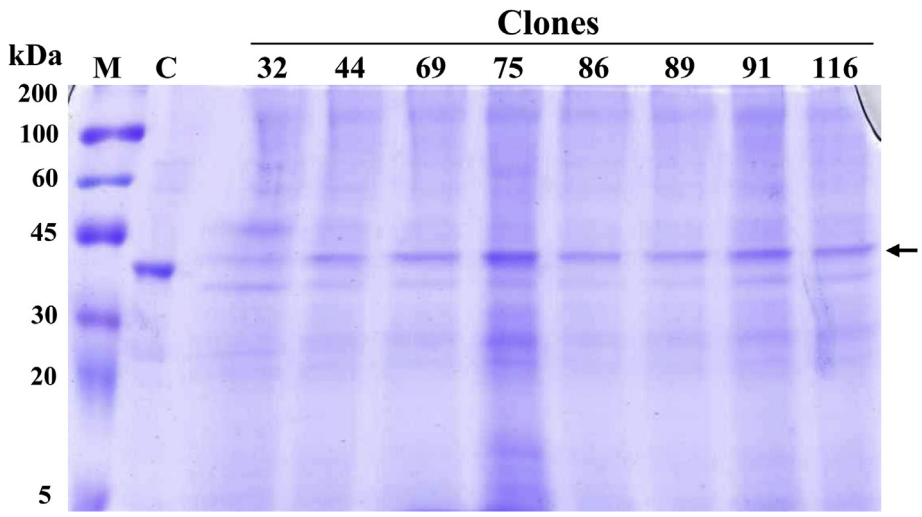

Fig. 3. Secreted protein profiles of chymosin-transformed clones of *Pichia pastoris*. Culture supernatants corresponding to 8 transformant clones with high level expression of recombinant chymosin after 144 h of methanol induction were evaluated by SDS-PAGE electrophoresis. The supernatant of *P. pastoris* clone 75, which exhibited the highest level of milk-clotting activity, showed the maximum quantity of heterologous chymosin. Recombinant chymosin was the most abundant protein secreted by the transformant clones, which is an advantage to achieve a successful purification. Lane M: protein molecular weight marker (kDa); Lane C: Commercial recombinant bovine chymosin; Lanes of clones: Culture supernatants of chymosin-transformed clones of *P. pastoris*. Arrow indicates the bands corresponding to recombinant bovine chymosin.

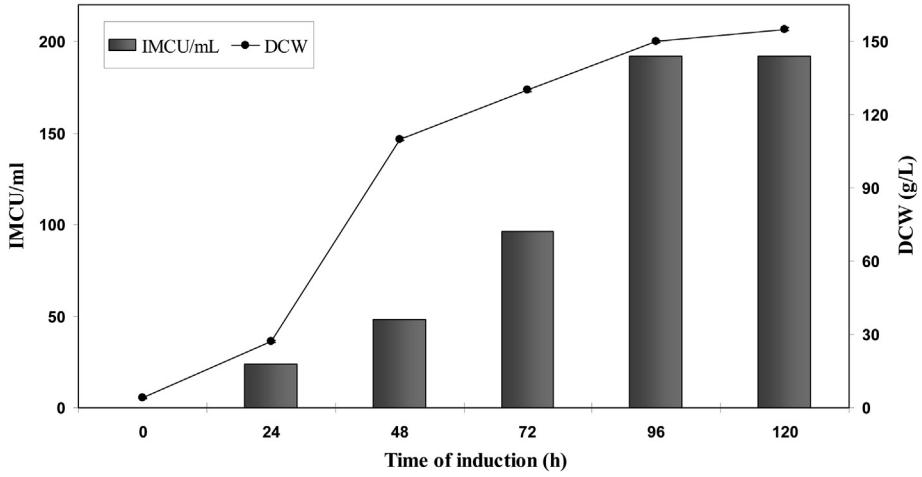

Fig. 4. Cell growth and recombinant bovine chymosin production through methanol induction in stirred-tank bioreactor. The transformant clone of *P. pastoris* that presented the highest level of recombinant chymosin expression (clone 75) was grown in a 6-L bioreactor applying a fermentation procedure with four phases. The evolution of biomass concentration (g DCW/L) and production of recombinant bovine chymosin (IMCU/ml) are shown during methanol induction fed-batch stage.

tangential microfiltration, anion exchange chromatography using Tricorn 10/50 column packed with DEAE-Sepharose was performed to separate recombinant bovine chymosin from other proteins. Several resins, pH values and salt concentrations were tested until the optimal purification conditions were achieved. Of note, this purification protocol was designed to avoid a previous desalting of the supernatant, by simply adjusting its pH value to 7 with 0.05 M sodium phosphate buffer. After injection of the sample into the column, UV_{280nm} line indicating protein concentration showed three spikes at 62 ml (second wash), 80 ml (fractions 3 and 4) and 107 ml (fraction 17), corresponding with addition of 14%, 20% and 100% of buffer B, respectively (Fig. 6A). Moreover, a small spike was detected at 94 ml of elution (fraction 10) which corresponded to 30% of buffer B (Fig. 6A). Fractions obtained from the chromatography were loaded in a polyacrylamide 12% gel to perform an SDS-PAGE and proteins were stained with Coomassie brilliant blue G250 (Fig. 6B). The binding of the protein to the column was very efficient since no chymosin was observed in the percolate. The first wash with buffer A, was unable to detach the protein, allowing reduction of this wash to a minimum volume. Subsequent wash with buffer B eliminated all contaminants detectable by the SDS-PAGE technique. A small amount of chymosin was lost in this step, indicating that further optimization by varying the percentage of buffer B to eliminate contaminants with minimum chymosin loss could be beneficial. Finally, as mentioned above, incremental addition of buffer B resulted in UV_{280nm} line spikes that corresponded to the recombinant chymosin which was detected in the SDS-PAGE as single bands (fractions 3, 4 and 17, Fig. 6B). Densitometry analysis of SDS-PAGE allowed establishing that the recombinant bovine chymosin in chromatography fraction 3 and 4 showed a 98 and 94% of purity, respectively; demonstrating that such enzyme was effectively purified from other proteins. On the other hand, fraction 10 contained few quantity of heterologous chymosin as was demonstrated by SDS-PAGE (Fig. 6B). At the end of the purification process,

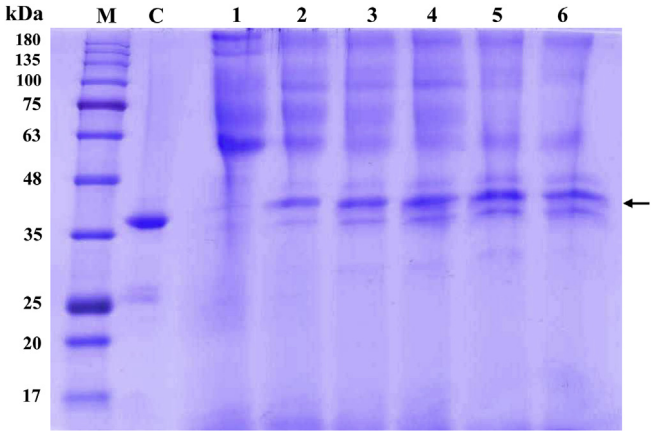

Fig. 5. Time course of recombinant bovine chymosin production by bioreactor fermentation. Production of recombinant bovine chymosin by fermentation in a stirred-tank bioreactor using a transformant clone with high expression level of chymosin (clone 75) was evaluated as a function of methanol induction time using SDS–PAGE electrophoresis. SDS–PAGE shows protein profiles from culture supernatants of different induction times. Lane M: protein molecular weight marker (kDa); Lane C: commercial recombinant bovine chymosin; Lane 1: start of methanol induction; Lane 2–6: 24, 48, 72, 96 and 120 h after methanol induction. Arrow indicates the bands of recombinant bovine chymosin.

chromatography fraction n°4 presented a milk-clotting activity of 384 IMCU/ml, an increase in specific activity of 54 fold and a total activity recovery of 48% (Table 1). The double band observed in fraction 4 has already been observed in our previous works [23,24], suggesting that the protein migration difference was due to the *N*-glycosylation sequon occupancy predicted by the software NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc). Further studies will be necessary to determine if the glycosylation hypothesis is correct and to verify whether the occupancy of that sequon affects clotting enzymatic activity, since the native chymosin is not glycosylated. Altogether, these results provide evidence that this protocol allows loading the column without previous desalting of the supernatant with appropriate purification yields of recombinant chymosin. It is worth mentioning the importance of being able to avoid the desalting step, given that basal media for *P. pastoris* usually contains high salt amounts and standard purification protocols need to include a diafiltration step followed by concentration of the protein, which impacts greatly in the final cost of the purified protein.

3.6. Temperature effect on recombinant bovine chymosin stability

The thermo-stability of recombinant bovine chymosin was analyzed at different storage temperatures from recombinant chymosin purified by ionic exchange chromatography. Consequently, we determined that milk-clotting activity was stable at a temperature of 5 °C throughout a period of 120 days (Fig. 7). However, coagulant activity decreased by half at 20 °C from 50 days of storage, that was maintained thereafter and reduced to undetectable values after 70 days of storage at 37 °C (Fig. 7). This study allowed establishing that the optimum temperature for the storage of unformulated recombinant chymosin is 5 °C, since clotting activity remained stable at this storage temperature throughout the whole evaluation period. These results suggest that the enzymatic activity of the unformulated recombinant chymosin can be kept stable at a refrigerator temperature without adding stabilizing and preserving compounds. On the other hand, we are currently evaluating the addition to the fermentation supernatants of some preservatives as potassium sorbate, and stabilizers such as glycerol, sodium chloride and trehalose, in order to prolong the stability over time of milk-clotting activity at temperatures above 5 °C.

3.7. Reiterative induction of recombinant chymosin expression in bioreactor

P. pastoris is one of the main yeasts expression systems currently used for the production of recombinant proteins at industry level due to the advantages described above [9,17]. However, when assessing productivity compared to other microorganisms used in industrial production such as *Escherichia coli*, results in a disadvantage as the bioprocess with *E. coli* can be 3–6 times faster than with *P. pastoris* depending on the conditions. In the case of chymosin, being an industrial enzyme, productivity becomes of great importance when considering cost schedule and feasibility of the production system. In this respect, we propose to overcome the low productivity obstacle by the recycling of biomass in the production process. After a complete bioreactor fermentation process, the supernatant was separated for recombinant chymosin purification and the cell biomass was reutilized for a subsequent methanol fed-batch feeding. After the first methanol induction phase, the reutilization of the biomass made it possible to reduce by half the time required for the subsequent reinductions while productivity (IMCU/ml h) was enhanced by 100%. Noteworthy, a second methanol reinduction period permitted to increase the productivity more than two folds. Regarding methanol-induction, when comparing the average productivity of standard condition (1.39 IMCU/ml h; day1-day5) to the average productivity of the whole procedure considering the initial induction plus the two reinductions (2.04 IMCU/ml h; day1-day3''), a productivity increment of 50% was achieved (Fig. 8). The cell biomass in the process of reinduction slightly fluctuates, showing a small loss in biomass level during pellet resuspension and afterwards a recovery of biomass concentration due to culture growth (Fig. 8). These results open the possibility of evaluating new techniques for reutilization of the biomass such as coupling the bioreactor to a tangential microfiltration system. This would probably diminish cellular stress, resulting in reduction of the decays in maximum yields of production after each methanol-reinduction period.

4. Conclusion

In the present work, a screening of *P. pastoris* clones transformed with the gene of bovine chymosin was carried out; which permitted to select a transformant clone with a milk-clotting volumetric activity 8 fold greater than the lowest value of the clones grown in basal salt medium. Noteworthy, although the level of recombinant-chymosin production is higher with YPD medium than with BSM, it is more convenient to use the saline medium because does not provide proteins to the culture and exhibit lower economic cost. Moreover, by SDS-PAGE we confirmed that the recombinant clone of *P. pastoris* which showed the highest milk-clotting activity achieved the maximum expression level of heterologous chymosin. The scaling of recombinant chymosin production was performed by a fed-batch strategy in stirred-tank bioreactor using biodiesel-derived crude glycerol as the carbon source and pure methanol for the induction of the heterologous protein expression. Such fermentation process allowed reaching a biomass concentration of 158 g DCW/L and a maximum milk-clotting activity of 192 IMCU/ml after 120 h of methanol induction. This value was 4-fold higher than that obtained in previous works where another chymosin-transformed clone was used [23,24]. The concentration of the total recombinant bovine chymosin expressed after 120 h of methanol induction in bioreactor with fed-batch strategy was 84 mg/L. Is important to mention that

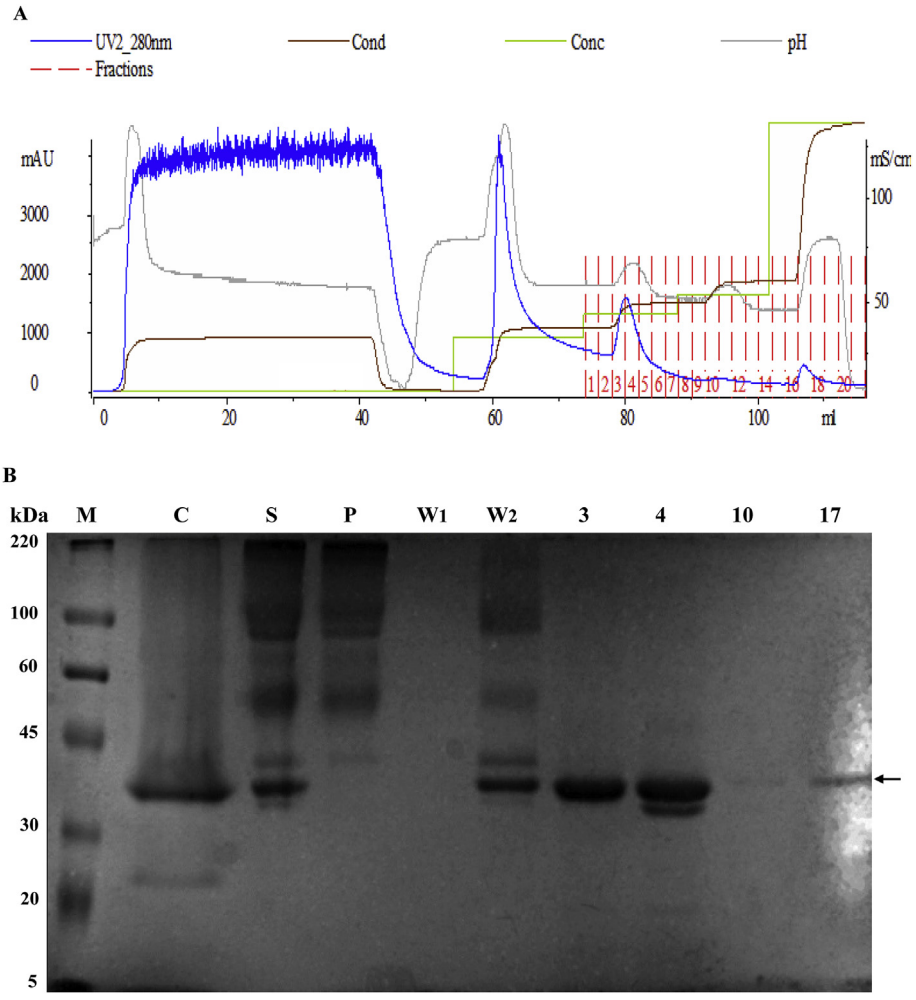

Fig. 6. Purification of recombinant chymosin by anion-exchange chromatography. A Tricorn 10/50 (GE) column packed with DEAE–Sepharose was utilized to perform the separation of recombinant bovine chymosin from other proteins and SDS-PAGE electrophoresis was used to analyze protein content of chromatography fractions. **(A)** Profile of the anion-exchange chromatography at an analytical scale of a bioreactor fermentation supernatant. *UV_280 nm*: Protein absorbance; *Cond*: Conductivity (mS/cm); *Conc*: Percentage of buffer B; pH: pH measurement; *Fractions*: elution fractions. (**B)** SDS-PAGE stained with Coomassie brilliant blue G250 to determine protein content in the different samples. *Lane M*: protein molecular weight marker (220, 100, 60, 45, 30, 20 and 5 kDa); *Lane C*: commercial recombinant bovine chymosin; *Lane S*: aliquot injected into column; *Lane P*: percolate; *Lane W1*: First wash of the column with buffer A (50 mM NaH_2PO_4 pH 7); *Lane W2*: Second wash of the column with addition of 14% of buffer B (2 M NaCl, 50 mM NaH_2PO_4, pH 7); *Lanes 3, 4, 10 and 17*: eluted fractions collected from the chromatographic run at elution volumes of 79 ml, 81 ml, 94 ml and 107 ml, respectively. Arrow indicates the bands of recombinant bovine chymosin.

Table 1
Purification process of recombinant bovine chymosin expressed by *P. pastoris* using fed-batch fermentation.

Process stage	Volume (ml)	Total Abs_{280}[a]	Milk-clotting activity			Increase in specific activity (fold)[e]	Total activity recovery (%)[f]
			Volumetric (IMCU/ml)[b]	Total (IMCU)[c]	Specific (IMCU/total Abs_{280nm})[d]		
Clarification	5	90	192	960	10.6	1	100
Concentration	1.5	55.5	576	864	15.57	1.5	90
Anion-exchange chromatography [g]	1.2	0.8	384	460	576	54	48

[a] Absorbance at 280 nm multiple by the volume.
[b] Milk-clotting activity in international milk clotting units per milliliter (IMCU/ml).
[c] Milk-clotting activity in IMCU/ml multiplied by the total volume.
[d] Specific activity expressed as the total milk-clotting activity (IMCU) divided by the total Abs_{280}.
[e] The specific activity of a stage divided by the specific activity of the cell-free supernatant.
[f] The total activity of a stage divided by the total activity of the cell-free supernatant and multiplied by 100.
[g] Chromatography fraction n°4.

the secreted recombinant prochymosin was activated at low pH during methanol-induction phase without the requiring of further chemical processing. Furthermore, the recombinant bovine chymosin was purified from the bioreactor-fermentation supernatant by anion-exchange chromatography which allowed obtaining active heterologous chymosin with high level of purity. This result

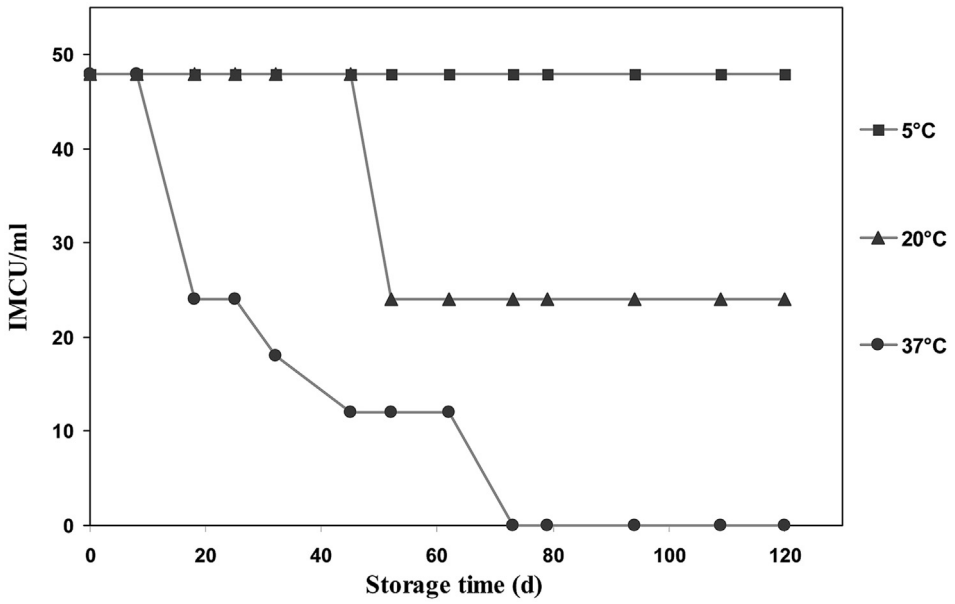

Fig. 7. Temperature effect on recombinant bovine chymosin stability. Thermo-stability of recombinant bovine chymosin was evaluated at different storage temperatures (5 °C, 20 °C and 37 °C). Milk-clotting activity kept stable at 5 °C through all the storage period (120 days), suggesting that this temperature is the optimal value for storing unformulated recombinant bovine chymosin produced by *P. pastoris* and purified by anion-exchange chromatography.

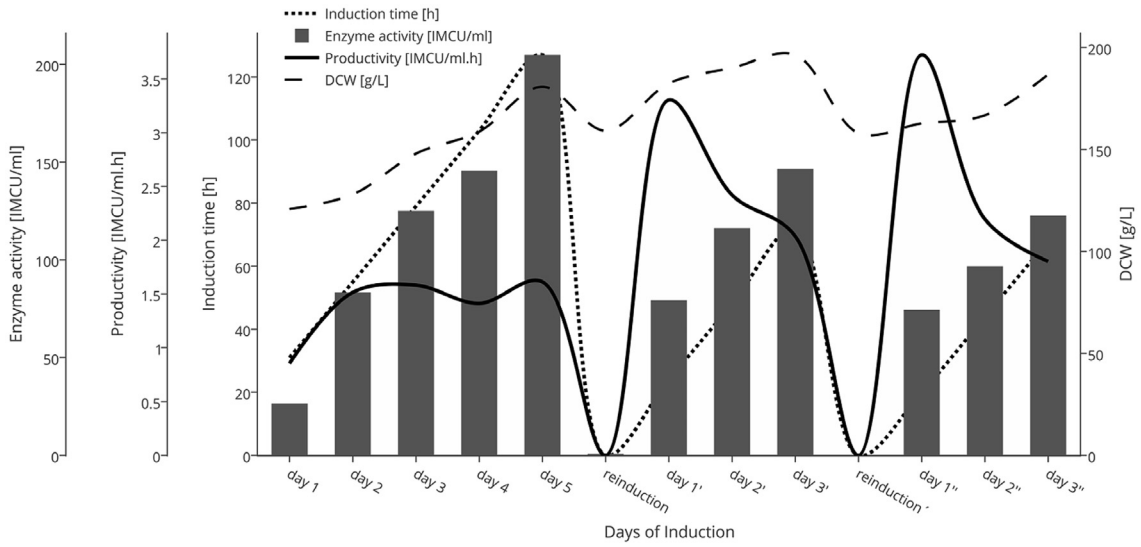

Fig. 8. Reiterative induction of recombinant chymosin expression in bioreactor. Reutilization of *Pichia pastoris* biomass was performed in order to achieve reiterative methanol inductions of recombinant bovine chymosin expression. During the first methanol reinduction phase the productivity was enhanced by 100% and in the second reinduction stage the productivity increased more than two folds. Cell biomass slightly fluctuates during reinduction process, showing a low decrease in biomass concentration during pellet resuspension and afterwards a recovery of biomass level due to culture growth.

should be remarked because such purification procedure can be scaled up in order to produce the enzyme at an industrial level. Moreover, we established that the optimum temperature for the storage of unformulated recombinant chymosin was 5 °C, which permitted to conserve the protein without the addition of stabilizing and preserving compounds during at least 120 days. Furthermore, reiterative methanol-inductions of recombinant chymosin expression in bioreactor generated an increase of the enzyme productivity of 50%, demonstrating that the reutilization of cell biomass overcame the low productivity usually achieved by *P. pastoris* system. As a general conclusion we demonstrated that from an intense screening of transformant clones of *P. pastoris* we were able to achieve a higher production of recombinant bovine chymosin by fermentation in bioreactor in comparison with previous studies. The high-purity heterologous chymosin obtained by anion-exchange chromatography suggests that this downstream procedure could be scaled up in a successful way. The recycling of biomass by reiterative methanol-inductions increased significantly the productivity of recombinant bovine chymosin.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by the PICT Start Up 2010 1662 grant from the National Agency for Science and Technology Promotion from the National Ministry of Science and Technology of Argentina and by UBACyT 2011–2014 101056 grant (issued to Miguel Angel Galvagno) and the grant 2010 SJ10/31 from the Universidad Nacional de San Martin (issued to Martin Blasco). We thank Gabriela Risso for her collaboration.

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