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Bioprocess and downstream optimization of recombinant bovine chymosin B in *Pichia* (*Komagataella*) *pastoris* under methanol-inducible *AOXI* promoter

Diego Gabriel Noseda [a,*], Martín Blasco [a], Matías Recúpero [a], Miguel Ángel Galvagno [a,b]

[a] Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico de Chascomús (IIB-INTECH), Universidad Nacional de San Martín (UNSAM) – Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), San Martín, 1650, Buenos Aires, Argentina
[b] Departamento de Ingeniería Química, Facultad de Ingeniería, Universidad de Buenos Aires, Pabellón de Industrias, Ciudad Universitaria, 1428, Buenos Aires, Argentina

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ABSTRACT

A clone of the methylotrophic yeast *Pichia pastoris* strain GS115 transformed with the bovine prochymosin B gene was used to optimize the production and downstream of recombinant bovine chymosin expressed under the methanol-inducible *AOXI* promoter. Cell growth and recombinant chymosin production were analyzed in flask cultures containing basal salts medium with biodiesel-byproduct glycerol as the carbon source, obtaining values of biomass level and milk-clotting activity similar to those achieved with analytical glycerol. The effect of biomass level at the beginning of methanol-induction phase on cell growth and chymosin expression was evaluated, determining that a high concentration of cells at the start of such period generated an increase in the production of chymosin. The impact of the specific growth rate on chymosin expression was studied throughout the induction stage by methanol exponential feeding fermentations in a lab-scale stirred bioreactor, achieving the highest production of heterologous chymosin with a constant specific growth rate of 0.01 h^{-1}. By gel filtration chromatography performed at a semi-preparative scale, recombinant chymosin was purified from exponential fed-batch fermentation cultures, obtaining a specific milk-clotting activity of 6400 IMCU/mg of chymosin and a purity level of 95%. The effect of temperature and pH on milk-clotting activity was analyzed, establishing that the optimal temperature and pH values for the purified recombinant chymosin are 37 °C and 5.5, respectively. This study reported the features of a sustainable bioprocess for the production of recombinant bovine chymosin in *P. pastoris* by fermentation in stirred-tank bioreactors using biodiesel-derived glycerol as a low-cost carbon source.

Introduction

Chymosin (EC3.4.23.4) is a specialized milk-clotting aspartic protease, which is produced by the abomasum mucosa of new born ruminants and other vertebrates during the first days of their life [1]. Particularly, bovine chymosin is the preferred enzyme for cheese manufacture since it specifically cleaves a Phe-Met bond in the κ-casein molecules of milks, reducing flavors in cheese that are generated as a result of non-specific cleavages [2]. Chymosin is expressed as a prepro-enzyme, from which the presequence of 16 amino acids is cleaved in the endoplasmic reticulum when the protein is secreted. Afterwards, the pro-sequence of 42 amino acids is cleaved autocatalytically at low pH producing the mature enzyme [3]. The production of bovine chymosin in microbial expression systems, as yeast and filamentous fungi present several advantages in comparison to the process used for obtaining the extraction-derived authentic chymosin [4–7]. The methylotrophic yeast *Pichia (Komagataella) pastoris* has become an important expression host for the production of active recombinant proteins [8,9]. The benefits of this expression system include growth to high biomass quantity on defined minimal medium [8,10], high expression level of heterologous proteins [11,12], typical eukaryotic post-translational modification, such as folding, glycosylation and disulfide bond formation [13,14], and the efficient secretion of extracellular proteins [15].

Moreover, *P. pastoris* has an efficient methanol-inducible promoter from the alcohol oxidase I gene (*AOX1*) which is commonly used for foreign gene expression [16]. A characteristic attribute of this promoter is that it is strongly repressed in the presence of some carbon sources, as glucose and glycerol, but induced over

* Corresponding author. Tel.: +54 11 4006 1500.
E-mail address: diegonoseda@yahoo.com.ar (D.G. Noseda).

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1000-fold with methanol as the sole carbon source, ensuring high cell concentration before the beginning of the methanol induction period [9,13]. Furthermore, *P. pastoris* preference for respiratory rather than fermentative metabolism, even at high-biomass processes, prevents the accumulation of secondary metabolites like ethanol and acetic acid [13]. On the other hand, *P. pastoris* is a GRAS organism [17,18], and is useful for scaling up heterologous protein production in different types of bioreactors [19].

In a previous work, we have cloned and expressed bovine chymosin B in *P. pastoris* strain GS115 under the control of the *AOX1* promoter, selecting a transformant clone with a notable expression level of recombinant chymosin, which is secreted and then activated by the acidity of the yeast culture [20]. We have also optimized and scaled up recombinant bovine chymosin production using a stirred-tank bioreactor obtaining high biomass level and milk-clotting activity [20].

In the present study, we analyzed the growth of a *P. pastoris* clone and the production of recombinant bovine chymosin in basal salt medium using biodiesel-derived crude glycerol as a low cost carbon source. Furthermore, the effect of biomass level at the start of methanol-induction phase on yeast growth and chymosin expression was established with the purpose to increase heterologous protein synthesis. Additionally, we optimized the recombinant bovine chymosin production by determining the optimal specific growth rate for chymosin expression during methanol fed-batch stage in a stirred-tank bioreactor. Moreover, we carried out semi-preparative chymosin purification by using molecular exclusion chromatography obtaining higher amounts of recombinant chymosin with an elevated degree of purity. Finally, purified recombinant bovine chymosin was employed for optimal temperature and pH determination for milk-clotting activity.

Materials and methods

Strains and reagents

A clone of *P. pastoris* (clone 1) transformed with bovine prochymosin B gene was utilized for the experimental studies. This clone was obtained from the selection method described in our previous work [20]. Commercial recombinant bovine chymosin (Maxiren-DSM; Heerlen, Netherlands) was used as the chymosin standard for milk clotting, SDS–PAGE and purification assays. Commercial powdered skimmed milk (Nestle; Vevey, Switzerland) was employed as the substrate for milk clotting evaluation.

Media composition

P. pastoris growth on solid medium was performed at 30 ± 1 °C using YPD medium containing (in grams per liter): peptone, 20; yeast extract, 10; glucose, 20 and agar, 20. Basal salts medium (BSM)[1] supplemented with trace metal solution (PTM1) and biotin, based on a previously described composition [21], was employed as the liquid culture medium for *P. pastoris* growth. Glucose, biodiesel-derived crude glycerol and methanol were used as carbon and energy sources depending the step of the experiment.

Biomass and glycerol determinations

The optical density of the culture samples was determined at 600 nm using an UV–Vis spectrophotometer and converted to dry cell weights (DCW, in g/L) with a previously calculated calibration curve according to the formula: $OD_{600nm} = 2.337 \times DCW$, $R^2 = 0.991$. Glycerol was measured in the cell-free supernatant samples from the different culture times by the microplate-adapted periodate method in accordance with Bondioli and Della Bella [22].

Milk-clotting assay

Milk-clotting activity was determined according to the end-point dilution assay [2,23]. Powdered skimmed milk utilized as the substrate was reconstituted at 26% (w/v) in 0.5 g/L $CaCl_2$ (pH 6.5), mixed at 25 ± 1 °C for 30 min and preincubated at 37 ± 1 °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 solution (50 μl) was added to each well containing the supernatant dilutions (50 μl). After stirring the mixtures, the plate was incubated at 37 ± 1 °C for 10 min and centrifuged at 2000*g* for 5 min. The milk-clotting activity was established using the highest dilution that induced milk coagulation, visualized as the formation of clots in the bottom of the wells. The highest dilution values were compared with those obtained with a solution of commercial recombinant bovine chymosin (600 IMCU/ml) to achieve the international milk clotting units per milliliter (IMCU/ml). Milk-clotting activity of each sample was evaluated in duplicate. As a negative control, blank culture medium was employed instead of the culture supernatant.

SDS–PAGE

Cell-free culture supernatants were mixed with cracking buffer and subjected to 12% SDS–PAGE according to Laemmli protocol [24]. The gels were stained with Coomassie brilliant blue (CBB) G-250 (Sigma–Aldrich; St. Louis, MO) to visualize the protein bands. Standard protein markers were used to estimate the molecular weight of the proteins from the supernatants. Recombinant bovine chymosin concentration in the culture supernatants and chromatography fractions was estimated by a calibration curve of BSA standard analyzed through SDS–PAGE and band densitometry using ImageJ software (http://rsb.info.nih.gov/ij).

P. pastoris growth and recombinant chymosin production using biodiesel-byproduct crude glycerol as the carbon source

Crude glycerol, the main byproduct of biodiesel industry, was utilized as the carbon and energy source of the culture medium to analyze the growth of *P. pastoris* for the subsequent production of recombinant bovine chymosin. For this purpose, crude glycerol derived from biodiesel industry was pretreated based on the description of Chi et al. [25]. Hence, the pH of the crude glycerol was adjusted to 6.0 with hydrochloric acid to convert the soluble soaps into insoluble free fatty acids which precipitated. The precipitate was separated from the crude glycerol by centrifugation at 3600*g* for 20 min. Accordingly, the content of glycerol was 88% v/v and 94% v/v before and after the mentioned treatment, respectively. *P. pastoris* clone 1 cells previously grown on YPD agar were employed to inoculate BSM (supplemented with 4 ml/L PTM1 and 30 μl/L biotin) containing biodiesel-derived crude glycerol (10 g/L) in Erlenmeyer flasks incubated on a rotary shaker at 250 rpm and 30 ± 1 °C. These cultures were sampled periodically during incubation for biomass level measurement, with the purpose of determinate kinetic parameters. After 24 h of cultivation, induction of bovine chymosin expression was initiated by adding 1% (v/v) pure methanol to the culture every 24 h of incubation for a period of 120 h. During methanol induction phase, flasks were incubated on shaker (250 rpm) at 25 ± 1 °C and with the addition of citrate–phosphate buffer pH 4 (final concentration: 0.1 M) every 24 h to maintain the optimal expression conditions, as was

[1] *Abbreviations used:* BSM, basal salts medium; DCW, dry cell weights; CBB, Coomassie brilliant blue; MWCO, molecular weight cutoff.

established by Noseda et al. [20]. Cultures were sampled every 24 h during the induction stage for cell density determination. Subsequently, culture samples were centrifuged at 3500*g* for 15 min at 4 °C, and the supernatants were used for measuring milk-clotting activity.

Effect of biomass level at the beginning of methanol induction phase on cell growth and recombinant chymosin expression

The optimal level of cellular biomass at the beginning of the methanol induction phase was determined using batch cultures of *P. pastoris* clone 1. Hence, cells grown on YPD agar were inoculated into 100-ml Erlenmeyer flasks containing 20 ml BSM with 10 g/L biodiesel-derived crude glycerol (supplemented with 4 ml/L PTM1 and 30 μl/L biotin) and cultured on shaker at 250 rpm and 30 ± 1 °C. After 24 h of incubation, cells were harvested by centrifugation at 3500*g* for 15 min and resuspended in flasks which contained BSM (supplemented with PTM1 and biotin) without glycerol so as to obtain three different levels of biomass: low level (1.5 g/L), middle level (3.2 g/L) and high level (5.8 g/L). Then, induction of bovine chymosin expression was started by adding 1% (v/v) pure methanol to the cultures every 24 h of incubation for 120 h. Throughout induction phase, flasks were incubated at 250 rpm and 25 ± 1 °C and with the addition of citrate–phosphate buffer pH 4 (final concentration: 0.1 M) every 24 h to sustain expression conditions. During this stage, cultures were sampled every 24 h with the purpose to measure cell density and milk-clotting activity.

Effect of specific growth rate on recombinant chymosin production during fermentation in bioreactor

The impact of different specific growth rates (μ) on recombinant bovine chymosin expression was analyzed during methanol induction phase in a stirred-tank bioreactor. The specific growth rates were maintained constant by an exponential feeding with pure methanol during the whole induction period. The exponential flow rate (F, in L/h) was calculated according to the following equation: $F(t) = (\mu V_o C_{Xo}/Y_{x/s}C_s)^{\mu t}$, where μ is the targeted specific growth rate, (h^{-1}); V_o, the initial volume (L); C_{Xo}, the initial cell concentration (g_x/L); $Y_{x/s}$, the cellular yield coefficient based on substrate consumption (g_x/g_s); C_s, the feed substrate concentration (g_s/L) and t, the induction time (h). The pre-fixed parameters in this stage were taken as: V_o = 4 L, $Y_{x/s}$ = 0.30 g_x/g_s and C_s = 800 g_s/L.

The bioreactor fermentation cultures were carried out in a four phase's process based on Celik et al. [26], with few modifications. The first stage consisted in a batch culture with BSM containing biodiesel-derived crude glycerol (40 g/L) as unique carbon source, in order to achieve high biomass level while repressing foreign gene expression. In the second phase, crude glycerol (600 g/L solution supplemented with 12 ml/L PTM1 and 90 μl/L biotin) was fed to the culture at a growth-limiting rate to further increase cell concentration and allowing a gradual de-repression of *AOX1* promoter [27]. In this period, glycerol feeding was performed by means of a constant feed profile, determined by 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_x/L); $Y_{x/s}$, the cellular yield coefficient based on substrate consumption (g_x/g_s); and C_s, the feed substrate concentration (g_s/L). In this period the pre-fixed parameters were taken as: μ_o = 0.16 h^{-1}, V_o = 3.2 L, $Y_{x/s}$ = 0.60 g_x/g_s and C_s = 600 g_s/L. Consequently, the constant feed flow throughout this phase was 0.045 L/h. Subsequently, a short transition stage was conducted by feeding with a crude glycerol:methanol (3:1) mixture, preparing the cells for the growth on methanol as the unique carbon source. Finally, the methanol induction phase was carried out by the addition of pure methanol (supplemented with 12 ml/L PTM1 and 90 μl/L biotin) using the exponential feeding profile described above with the following values of specific growth rates: 0.005, 0.01 and 0.02 h^{-1}. The initial biomass concentration in this stage was 120 g DCW/L. The inoculums for the fermentations were obtained from *P. pastoris* clone 1 cells grown on YPD agar. The cells were inoculated into a 100-ml flask containing 20 ml YPD broth and cultured overnight at 30 ± 1 °C and 250 rpm. Then, 200 ml of BSM with 40 g/L glycerol (supplemented with 4 ml/L PTM1 and 30 μl/L biotin) in a 1-L flask was inoculated with the overnight culture and incubated at 30 ± 1 °C and 250 rpm until the culture reached an OD_{600} of ~25. This culture was employed to inoculate 3 L of the aforementioned basal saline medium. Fermentations were conducted in a 7-L Bio-Flo 110 stirred-tank bioreactor (New Brunswick Scientific; Edison, NJ) interfaced with Biocommand Bioprocessing software (New Brunswick Scientific) used for data acquisition and parameter control. The temperature was maintained at 30 ± 1 °C through batch, glycerol fed-batch and transition phases, and at 25 ± 1 °C during methanol-induction period as was previously established [20]. A pH value of 5 was hold throughout the first three stages, and a pH of 4 was kept in the last phase; these values were controlled through the addition of 85% (v/v) H_3PO_4 and 25% (v/v) NH_4OH. The dissolved oxygen was maintained above 30% of saturation and controlled by agitation cascade and filter-sterilized air. The pH was monitored online using a pH electrode (Mettler-Toledo GmbH, Germany) and the oxygen concentration was measured with a polarographic probe (InPro6110/320, Mettler-Toledo GmbH). Foam generation was avoided by the addition of 0.3% (v/v) antifoam 289 (Sigma–Aldrich). Fermentation cultures were sampled during methanol-induction phase to evaluate biomass level and milk-clotting activity. This study was carried out by performing 3 fermentation processes for each specific growth rate previously mentioned.

Semi-preparative scale purification of recombinant bovine chymosin

A bioreactor fermentation culture corresponding to a 96 h fed-batch methanol feeding conducted with a constant specific growth rate of 0.01 h^{-1} was centrifuged at 3500*g* for 15 min, and the supernatant used as recombinant bovine chymosin source. A volume of 10 ml of cell-free supernatant was subjected to fast ultrafiltration in order to concentrate the recombinant bovine chymosin. For this purpose, an Amicon Ultra-4 device (Millipore Corp., Billerica, MA) with a molecular weight cutoff (MWCO) of 3 kDa was utilized at 7500*g* for 40 min and 5 °C. Then, the concentrated supernatant was applied to a Superdex 75 HR 10/30 prepacked column (Pharmacia Biotech Inc., Piscataway, NJ) connected to a FPLC system to carry out high performance gel filtration chromatography at a semi-preparative scale. The column was equilibrated with two column volumes of a buffer consisting of 0.05 M NaH_2PO_4 and 0.15 M NaCl at pH 7.0 and the elution was performed with such buffer at a flow rate of 0.25 ml/min. The chromatography fractions were evaluated to determine the volumetric milk-clotting activity. The fraction exhibiting coagulation activity was concentrated with a 3000 MWCO device as was described above. The concentrated fraction was analyzed by 12% SDS–PAGE with the purpose to compare its protein profile with that obtained from the fermentation culture supernatant. Total protein content was estimated by measuring the absorbance at 280 nm using a Beckman spectrophotometer. A commercial recombinant bovine chymosin was also examined by gel filtration chromatography applying the mentioned conditions with the aim to establish the elution time for bovine chymosin. Recombinant chymosin content was estimated by SDS–PAGE and band densitometry as was explained before.

Determination of optimum temperature and pH for recombinant chymosin activity

The effect of temperature on milk-clotting activity was evaluated from 20 to 45 °C. Briefly, the enzyme activity of the recombinant bovine chymosin purified by chromatography was measured by the end-point dilution assay at different temperatures of the mentioned range. Each tested incubation temperature was analyzed in triplicate. The pH effect on milk-clotting activity was studied at 37 °C, in acetate buffer with pH values ranging from 4.5 to 7. For this, purified recombinant bovine chymosin was tested by the end-point dilution assay in triplicate for each analyzed value.

Results and discussion

Cell growth and chymosin production with biodiesel byproduct crude glycerol as carbon source

Erlenmeyer's flasks cultures of *P. pastoris* clone 1 in BSM containing biodiesel-derived crude glycerol displayed a maximum specific growth rate (μ_{max}) of 0.16 h^{-1} and a biomass yield coefficient ($Y_{x/s}$) of 0.62 g DCW/g of crude glycerol (Fig. 1). At the end of this batch phase (24 h of incubation), the culture reached a biomass level of 6.15 g DCW/L and glycerol was completed consumed. Furthermore, during the methanol induction phase *P. pastoris* culture continued growing with an average specific growth rate (μ) of 0.03 h^{-1}. After 96 h of induction the culture attained a maximum cell concentration of 12.6 g DCW/L with a biomass yield coefficient ($Y_{x/s}$) of 0.27 g DCW/g of methanol (Fig. 1). It is noteworthy that the kinetic and stequiometric values achieved in both phases are similar to those obtained from *P. pastoris* clone 1 cultures performed in BSM containing analytical glycerol as the carbon source for the growing phase, as was described in our previous work [20]. The volumetric milk-clotting activity increased during this period reaching its maximum level (3 IMCU/ml) from 84 h after the start of methanol induction (Fig. 2). This result is consistent with that obtained from *P. pastoris* clone 1 grown in BSM containing analytical glycerol as was also reported in Noseda et al. [20]. Thereby, we demonstrated that analytical glycerol can be replaced by biodiesel-derived crude glycerol to be used as the carbon source for an appropriate growth of *P. pastoris* cells and the subsequent production of recombinant bovine chymosin by methanol feeding. Importantly, this result agrees with previous reports that used

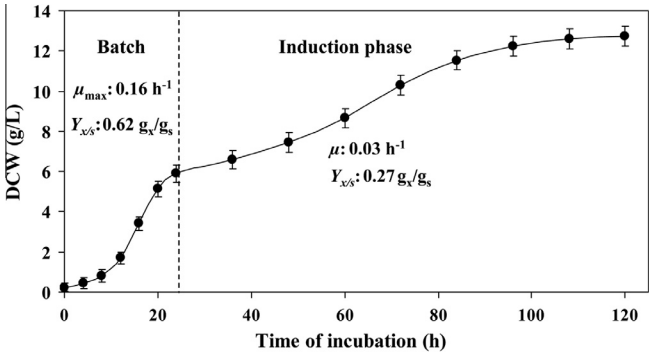

Fig. 1. Growth of *P. pastoris* clone 1, transformed with bovine chymosin gene, through batch phase in basal salts medium containing biodiesel-derived crude glycerol as the unique carbon source and during the induction stage of chymosin expression carried out by methanol addition. The profile of biomass level among *P. pastoris* growth is expressed in dry cell weight (DCW, in g/L). Maximum specific growth rate (μ_{max}, in h^{-1}) and a biomass yield coefficient ($Y_{x/s}$, in g of DCW/g of substrate) are indicated for batch and methanol-induction phases.

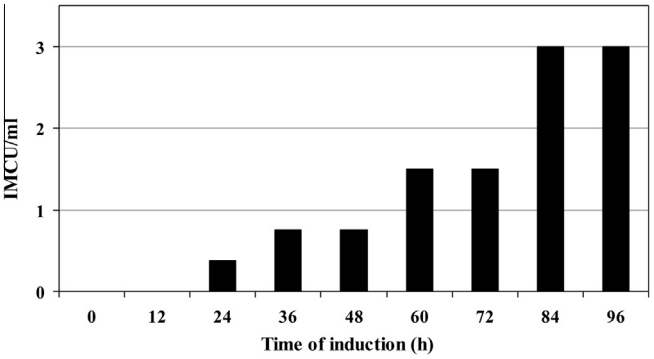

Fig. 2. Production of recombinant bovine chymosin during methanol induction of *P. pastoris* clone 1 grown in basal salts medium containing biodiesel-derived crude glycerol. Milk-clotting activity is expressed in international milk clotting units per milliliter (IMCU/ml). Recombinant bovine chymosin activity increased during methanol-induction phase reaching a maximum level after 84 h of induction.

biodiesel-byproduct crude glycerol as the sole carbon source of the culture media for the production of other recombinant proteins [28].

Effect of biomass level at the beginning of induction on cell growth and chymosin expression

The optimal level of biomass at the start of methanol induction phase was determined from methanol-induced batch cultures in shake flasks by means of the evaluation of different cell concentrations. Therefore, we determined that a high level of biomass (5.8 g/L) at the start of the induction stage generated an increase in the production of recombinant bovine chymosin because a higher concentration of cells is reached at the end of this period (Fig. 3). In the other hand, with middle and low initial levels of biomass (3.2 g/L and 1.5 g/L, respectively), cell growth is lower throughout methanol induction phase which leads to a minor bovine chymosin expression. In this way, we confirmed that the amount of recombinant bovine chymosin produced by *P. pastoris* is proportional to cell density at the beginning of induction stage. Noteworthy, the specific growth rates (μ) achieved during this period were similar for the three levels of biomass that were analyzed. These results are in agreement with previous studies of production of different heterologous proteins using this type of expression system, where the level of recombinant protein expression depends on the initial biomass concentration [29,30]. It is important to mention that, these values are critical for planning the production of heterologous proteins by fermentation processes in stirred-tank bioreactors.

Effect of specific growth rate on recombinant chymosin expression in bioreactor-scale fermentation

The production of recombinant bovine chymosin was analyzed as a function of the constant specific growth rates (μ) by an exponential feeding of methanol in bioreactor fermentation cultures. The highest production of recombinant bovine chymosin was achieved after 96 h of pure methanol induction with a constant specific growth rate of 0.01 h^{-1} throughout the protein expression period (Fig. 4). It is noteworthy that, the value of milk-clotting activity achieved by this feed strategy (96 IMCU/ml) was two times higher compared to that obtained by constant-flow strategy, which was reported in our previous work [20]. Using specific growth rates higher (0.02 h^{-1}) and lower (0.005 h^{-1}) than 0.01 h^{-1}, resulted in a noticeable decreased of the recombinant chymosin production. During methanol induction phase, the recombinant bovine chymosin levels kept increasing despite of the specific growth rate (data

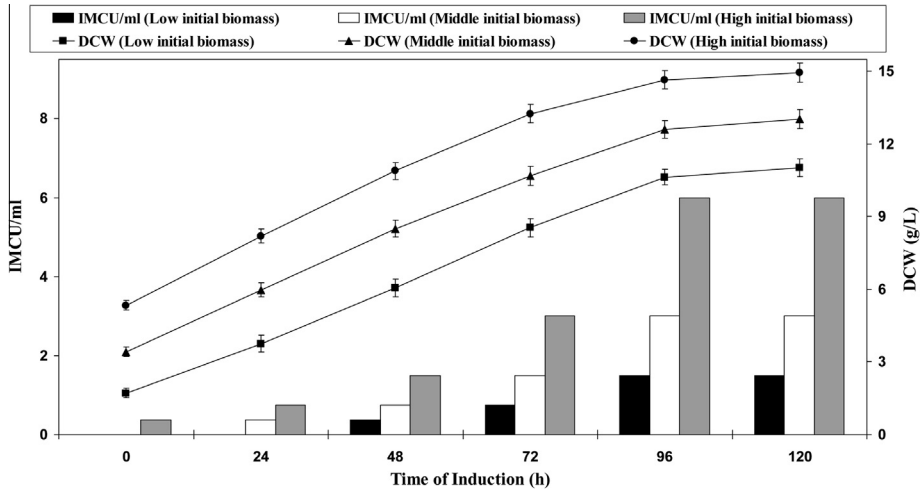

Fig. 3. Effect of biomass level at the beginning of methanol-induction phase on cell growth and recombinant bovine chymosin expression. Cell density and clotting activity are expressed in dry cell weight (DCW, in g/L) and international milk clotting units per milliliter (IMCU/ml), respectively.

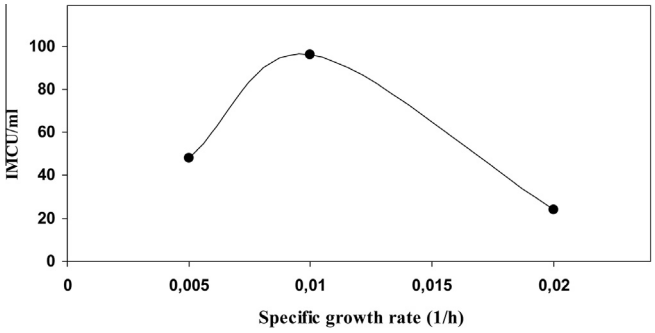

Fig. 4. Effect of specific growth rate on recombinant bovine chymosin production in bioreactor-scale fermentation. Three constant specific growth rates were evaluated during methanol-induction phase by an exponential feeding strategy in a stirred-tank bioreactor. Recombinant bovine chymosin activity is expressed in international milk clotting units per milliliter (IMCU/ml).

not shown), which suggest that enzyme degradation not occurred. With respect to the cell growth, it was observed that a specific growth rate of 0.01 h^{-1} allowed the culture to achieve a biomass level of 154 g DCW/L, while with growth rates of 0.005 and 0.02 h^{-1} the cell concentration was 138 and 172 g DCW/L, respectively. These confirm that high levels of biomass at the end of the bioreactor-fermentation process do not necessarily generate high amounts of heterologous protein. Thus, this results indicated that the relationship between the expression of recombinant bovine chymosin and the specific growth rates during induction phase is non-linear, which is in accordance with other studies of recombinant protein production in *P. pastoris* [31,32].

Purification of recombinant bovine chymosin at a semi-preparative scale

The recombinant bovine chymosin produced by methanol fed-batch fermentation in stirred bioreactor was purified from the culture supernatant (1745 IMCU/mg of chymosin) by a procedure that include high performance gel filtration chromatography at a semi-preparative scale. The cell-free supernatant was 4-fold concentrated by fast ultrafiltration, removing salts, residual methanol as well as peptides and small proteins. The semi-preparative scale chromatography permitted an appropriate separation of the proteins synthesized by *P. pastoris*, getting a distinctive fraction profile (absorbance at 280 nm as a function of the elution volume) that is

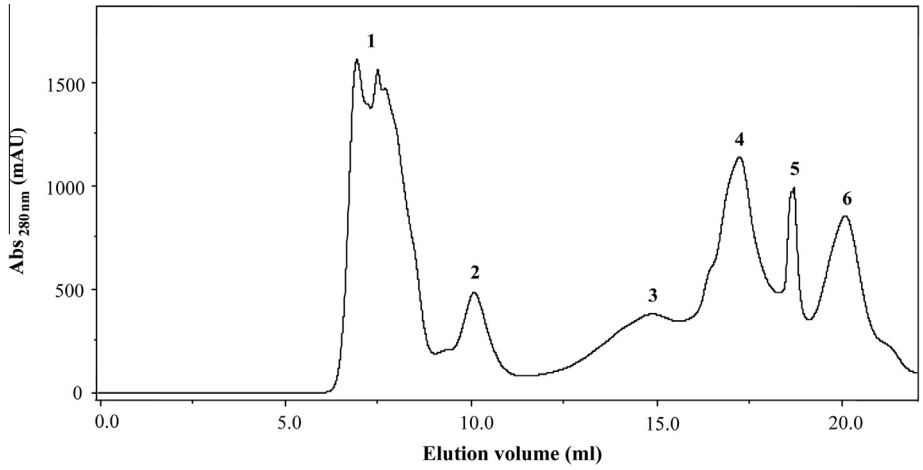

Fig. 5. Profile of a semi-preparative gel filtration chromatography of a bioreactor fermentation supernatant analyzed by a Superdex 75 HR 10/30 prepacked column. Numbered peaks correspond to the chromatogram fractions which were analyzed by the milk-clotting assay. The fraction of the peak No 2 (elution volume of 10 ml) was the only one that presented milk-coagulation activity. The elution volume of peak No 2 is analogous with the corresponding to the standard of recombinant bovine chymosin.

Table 1
Purification and concentration procedure of recombinant bovine chymosin produced in *Pichia pastoris* by 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_{280})[d]		
Cell-free supernatant	10	85	96	960	11.3	1	100
3 kDa fast ultrafiltration	2.65	50.4	384	1018	20.2	2	106
High performance gel filtration chromatography	2.35	0.49	192	451	914	81	47
3 kDa fast ultrafiltration	0.85	0.38	768	653	1707	151	68

[a] Absorbance at 280 nm multiplied 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.

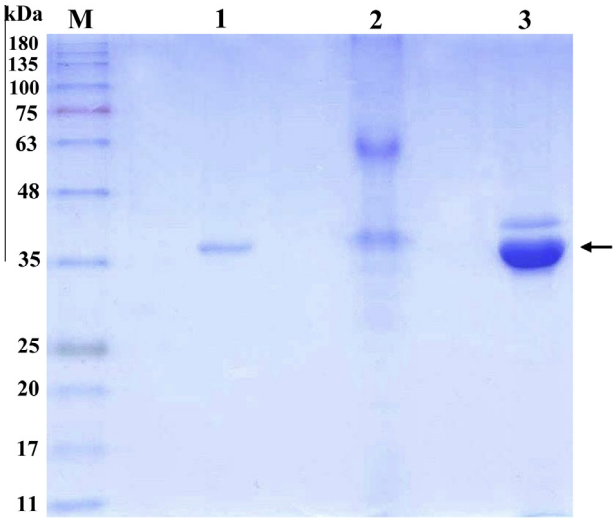

Fig. 6. SDS–PAGE analysis from the semi-preparative scale purification process. *Lane M*: protein molecular weight marker (kDa); *Lane 1*: commercial recombinant bovine chymosin; *Lane 2*: bioreactor fermentation cell-free supernatant; *Lane 3*: Gel-filtration chromatography fraction concentrated by fast ultrafiltration. A 20-μl volume was loaded for each sample. Arrow indicates the bands of recombinant bovine chymosin.

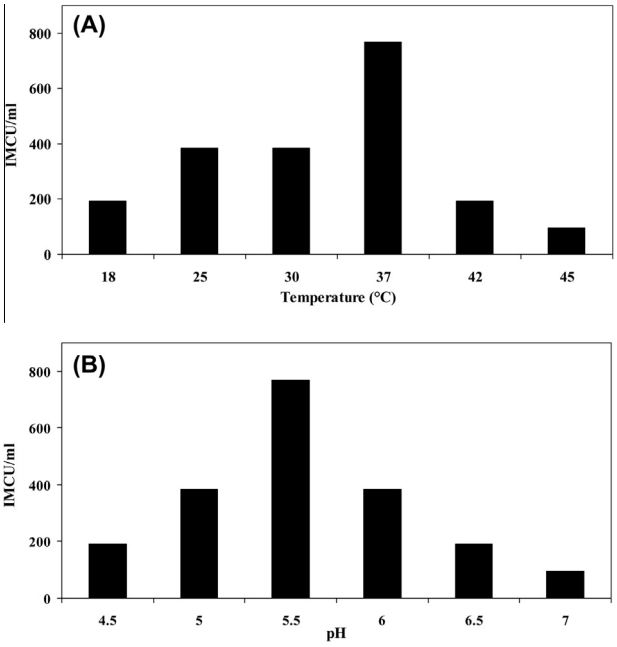

Fig. 7. Effects of temperature and pH on milk-clotting activity of the purified bovine chymosin. Recombinant enzyme activity was evaluated at different values of temperature (A) and pH (B) using skim milk as substrate applying the end-point dilution assay.

showed in Fig. 5. The chromatography fractions were analyzed by the milk-clotting assay determining that fraction No 2, which eluted with a retention volume of 10.1 ml, was the only one that exhibited milk-coagulation activity. Then, this fraction was 4-fold concentrated by fast ultrafiltration which presented a volumetric milk-clotting activity of 768 IMCU/ml and a total activity recovery of 68% (Table 1). The increase of this value could be due to the elimination of inhibitors or proteases of bovine chymosin. Furthermore, SDS–PAGE analysis indicated that such chromatography fraction contained recombinant bovine chymosin as the main protein with a 95% of purity (Fig. 6), demonstrating that the enzyme was effectively purified from other proteins expressed by *P. pastoris* clone 1. Also, we determined that by this purification and concentration process 120 mg/L of recombinant bovine chymosin was obtained at the end of the procedure which corresponds to a specific activity of 6400 IMCU/mg of chymosin. The recombinant bovine chymosin purified by the mentioned chromatography method was employed for establishing the optimum temperature and pH values of the milk-clotting activity.

Optimum temperature and pH for clotting activity

Milk-clotting activity profile at different temperatures indicated that 37 °C is the optimal reaction value for the recombinant bovine chymosin produced by *P. pastoris* clone 1 (Fig. 7A). Temperatures values lower and higher than 37 °C showed lower levels of milk-coagulation activity. On the other hand, we determined that a pH of 5.5 was the optimal to achieve the highest coagulant activity with the recombinant chymosin expressed by *P. pastoris* clone 1 (Fig. 7B). It should be mentioned that, with pH values lower and higher than 5.5 lesser milk-clotting activity was observed. These results are consistent with those obtained with the standard of recombinant bovine chymosin (data not shown). Thus, through the application of a temperature of 37 °C and a pH value of 5.5, an optimal milk-clotting activity could be obtained in cheese manufacture process with the utilization of the recombinant bovine chymosin produced by *P. pastoris* clone 1.

Conclusion

In the present study, a clone of *P. pastoris* transformed with bovine prochymosin B gene was grown in basal saline medium containing biodiesel-byproduct crude glycerol; demonstrating that this substrate could substitute analytical glycerol as the energy and carbon source for a proper cell growth and the concomitant expression and secretion of active bovine chymosin. It is important to notice that crude glycerol will remarkably reduce the process cost for the production of recombinant bovine chymosin, which makes it a sustainable biotechnological procedure. Furthermore, in this work we demonstrated that the amount of recombinant bovine chymosin expressed by *P. pastoris* is proportional to biomass level at the beginning of methanol-induction period. Moreover, a constant specific growth rate of 0.01 h^{-1} during protein expression phase resulted in a higher recombinant bovine chymosin production after 96 h of methanol induction. It is noteworthy, that all the results obtained are important for designing the production of recombinant bovine chymosin by methanol fed-batch fermentation strategies using stirred-tank bioreactors. Furthermore, the semi-preparative purification performed by gel filtration chromatography allowed obtaining a high amount of recombinant bovine chymosin with an elevated level of purity. Finally, we determined that the optimal temperature and pH values for the purified recombinant bovine chymosin are 37 °C and 5.5, respectively. In conclusion, a sustainable process based on the use of crude glycerol derived from biodiesel industry could be applied for the optimized production of recombinant bovine chymosin in *Pichia pastoris* by exponential fed-batch fermentation with stirred-tank bioreactors. This recombinant milk-clotting enzyme could be used in the elaboration of diverse types of cheeses due its efficiency in coagulating the caseins of milks from different sources.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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