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

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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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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).