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Cloning and Expression of Buffalo Active Chymosin in *Pichia pastoris*

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To date, only recombinant chymosin has been obtained in its active form from supernatants of filamentous fungi, which are not as good candidates as yeasts for large-scale fermentations. Since *Bos taurus* chymosin was cloned and expressed, the world demand for this protease has increased to such an extent that the cheesemaking industry has been looking for novel sources of chymosin. In this sense because buffalo chymosin has properties that are more stable than those of *B. taurus* chymosin, it may occupy a space of its own in the chymosin market. The main objective of the present work was the production of active recombinant buffalo chymosin in the culture supernatant of *Pichia pastoris*. This yeast has demonstrated its usefulness as an excellent large-scale fermentation tool for the secretion of recombinant foreign proteins. RNA was extracted from the abomasum of a suckling calf water buffalo (*Bubalus arnee bubalis*). Preprochymosin, prochymosin, and chymosin DNA sequences were isolated and expressed into *P. pastoris*. Only the recombinant clones of *P. pastoris* containing the prochymosin sequence gene were able to secrete the active form of the chymosin to the culture supernatant. This paper describes for the first time the production of active recombinant chymosin in *P. pastoris* without the need of a previous in vitro activation. The new recombinant yeast strain could represent a novel and excellent source of rennet for the cheesemaking industry.

KEYWORDS: Active chymosin; *Bubalus arnee bubalis*; expression; *Pichia pastoris*

INTRODUCTION

The rennets usually employed in the cheesemaking industry basically consist of aspartic proteases (*1*). These proteases contain two essential aspartic acid residues, Asp^{32} and Asp^{215}, at the active site. Their bilobulate three-dimensional conformation, where the active site is located between both lobules, confers on them the ability to act against the $Phe^{105}-Met^{106}$ peptide bond present in milk *k*-casein molecules, originating insoluble *para*-κ-casein, which finally leads to milk coagulation (*2*). These proteases have been extensively used for the traditional manufacture of milk curds as a precursor step for the elaboration of different types of cheeses, chymosin being the most efficient enzyme for the cheesemaking industry (*3*). Another aspartic protease employed in the industry may originate nonspecific digestions of milk caseins, causing bitter flavors and losses of protein content, which may lead to an important decrease in yield during the cheesemaking process (*4*).

Chymosin is synthesized in vivo as preprochymosin, which contains a signal peptide of 16 amino acid residues that is removed to generate prochymosin. This molecule is then processed to active chymosin under the acidic pH conditions present in the stomach, where a 42 amino acid propeptide located at the amino terminal end is removed (*5*).

Currently, cheesemaking industries use sources of rennet of four different origins: animal, plant, microbial, and recombinant rennet. Animal rennet, chymosin, has been traditionally obtained by direct pressing of the abomasum of suckling calves. This rennet, however, cannot cover the current demands of the world market and, in addition, its use may be risky owing to the appearance of infectious agents or impurities. Plant rennet has been obtained from flowers such as *Cynara cardunculus* (*6*), restricted to certain geographic areas and used for the production of special types of cheese. Microbial rennet has been obtained from the culture supernatants of microorganisms such as *Endothia* (*7*) or *Rhizomucor* (*8*). This type of rennet contains low specific proteases that may lead to bitter flavor in the products and low curd yields during the cheesemaking process (*9*). Finally, commercialized recombinant rennet is currently obtained from recombinant microorganisms containing the *Bos taurus* chymosin (*10, 11*). Yeasts such as *Saccharomyces cerevisiae* (*12*) or *Kluyveromyces lactis* (*13, 14*) and filamentous fungi such as *Trichoderma reesei* (*15*) or *Aspergillus oryzae* (*16*) have been used to produce recombinant chymosin, but only

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Table 1. Oligonucleotides Used in This Work

oliglonucleotide	oligonucleotide sequence	source for oligonucleotide design[a]
preprochym	5′-ATGAGGTGTCTCGTGGTGCTACTT-3′	*B. taurus* chymosin (**NM_180994**)[b]
prochym	5′-ATGGCAGAAATCACCAGAATCCCTC-3′	*B. arnee bubalis* chymosin (**AF177290**)[b]
chym	5′-ATGGGTGAGGTAGCGAGCGTGCC-3′	*B. arnee bubalis* chymosin (**AF177290**)[b]
chymosin	5′-TCAGATAGCCTTCGCCAGCCC-3′	*B. arnee bubalis* chymosin (**AF177290**)[b]
PGAP forward	5′-GTCCCTATTTCAATTGAA-3′	pGAPZαA (Invitrogen)
3′ AOX	5′-GCAAATGGCATTCTGACATCC-3′	pGAPZαA (Invitrogen)

[a] Genbank accession code. [b] See Supporting Information.

filamentous fungi were able to secrete active chymosin into the supernatant. However, filamentous fungi are not as good candidates as yeasts for performing large-scale fermentations (*17*).

The recombinant chymosin world demand increases yearly as shown by the number of patents recently requested. In fact, in the past few years goat chymosin (*14*) or camel chymosin (*18*) produced by microorganisms has been proposed. Buffalo chymosin shows physicochemical properties more stable than those of *B. taurus* chymosin (*19*), and their amino acid sequences show an identity of 97%. At the same time, *Pichia pastoris* is an excellent secretor of heterologous proteins into the culture medium. Because *P. pastoris* produces few extracellular endogenous proteins, the production of an interesting protein in this yeast may be considered as an initial step of the recombinant protein purification process (*17*). In addition, *P. pastoris* has demonstrated its usefulness as a large-scale fermentation tool for the production of recombinant foreign proteins (*17*) and, therefore, has been chosen as the best host for the expression of buffalo chymosin. Here we have developed for the first time a yeast able to secrete active chymosin into the supernatant. This recombinant strain offers a novel alternative for the production of recombinant chymosin.

MATERIALS AND METHODS

Strains Plasmids and Culture Conditions. The strains employed were *Escherichia coli* TOP10 and *P. pastoris* GS115 (both from Invitrogen, Carlsbad, CA).

pCR Blunt II TOPO vector (Invitrogen) was used to clone blunt-ended preprochymosin, prochymosin, and chymosin genes. pGAPZαA vector (Invitrogen) was used to express the genes in *P. pastoris*.

E. coli TOP10 cells were cultured in LB medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) supplemented with kanamycin (50 μg/mL) or Zeocin (25 μg/mL) to select transformants. *P. pastoris* GS115 was cultured in YPD medium (10 g/L yeast extract, 10 g/L peptone, and 20 g/L glucose) supplemented with Zeocin (Invitrogen) (100 g/mL) to select transformant yeast cells.

Isolation and Cloning of the Preprochymosin, Prochymosin, and Chymosin Genes. An abomasum of a male suckling calf was obtained from a slaughterhouse. The tissue was then cooled in liquid N_2 and stored at −80 °C. A tissue sample of 30 mg powdered and submerged in 300 μL of RNA later stabilization reagent (Qiagen, Valencia, CA). RNA was isolated using the RNeasy Plant Mini Kit (Qiagen), following the instructions recommended by the manufacturer. RT-PCR assays were performed to obtain cDNA molecules corresponding to preprochymosin, prochymosin, and chymosin, respectively. Three oligonucleotide pairs were designed from the *B. taurus* preprochymosin sequence (Genbank accession code NM_180994) and the *Bubalus arnee bubalis* prochymosin sequence (Genbank accession code AF177290) (**Table 1**) and used to carry out RT-PCR. The first cDNA strand was synthesized using first strand cDNA synthesis kit for RT-PCR AMV (Roche Applied Science, Indianapolis, IN). Second cDNA strand synthesis was carried out by PCR using the above-mentioned oligonucleotides. The reaction mixture contained polymerase buffer, 0.2 mM of each oligonucleotide, 1.5 mM of $MgCl_2$, 0.2 mM of each deoxynucleotide, and 1 unit of Accuzyme DNA polymerase (Bioline, Taunton, MA). PCR reactions were carried out using the following program: 96 °C for 2 min, 35 cycles of 96 °C for 1 min; 57 °C for 1 min and 72 °C for 2 min; and a final extension of 72 °C for 5 min. The three PCR products were cloned into the pCR Blunt II TOPO vector (Invitrogen) employing the Zero Blunt TOPO PCR Cloning Kit (Invitrogen). DNA inserts were then sequenced using the universal oligonucleotides SP6 and T7.

Integration of the Preprochymosin, Prochymosin,and Chymosin Genes into the *P. pastoris* Genome. The pGAPZαA vector was used to express preprochymosin, prochymosin, and chymosin genes in *P. pastoris*. The three sequences, obtained from *Eco*RI digestion of the previous pCR Blunt II TOPO constructions, were cloned into the pGAPZαA vector previously digested with the same restriction enzyme. The constructions obtained were used to transform *E. coli* TOP10 competent cells. The enzyme *Kpn*I was employed to analyze the orientation of the inserts with respect to the pGAP promoter. Vectors containing the inserts properly oriented were then linearized using the *Bsp*HI restriction enzyme, after which they were introduced into *P. pastoris* by electroporation. To obtain electrocompetent *P. pastoris* cells, 5 mL of medium was first inoculated and incubated at 30 °C overnight. This culture was used to inoculate 500 mL of medium, which was maintained at 30 °C until the OD_{600nm} reached a value between 1.3 and 1.5. The culture was then centrifuged at 1500*g* for 5 min at 4 °C, and the cells were resuspended in 500 mL of cool sterile water. After a second centrifugation, the cells were resuspended in 20 mL of cool 1 M sorbitol and finally centrifuged again and resuspended in 1 mL of cool 1 M sorbitol. To carry out the electroporation, 10 μg of the linearized plasmid was added to 80 μL of electrocompetent cells. The mixture was incubated on ice in the electroporation cuvette (Pulser cuvette, 0.2 cm) and subjected to a 1500 V, 200 Ω, and 25 μF pulse. Immediately, 1 mL of cool 1 M sorbitol was added, and the whole content was transferred to a 10 mL sterile tube and incubated at 30 °C for 2 h without shaking. Finally, the cells were spread onto Zeocin (Invitrogen) plates.

Integration of the chymosin gene into the *P. pastoris* genome was investigated by PCR using genomic DNA extracted from *P. pastoris* by means of the Lyse-N-Go PCR Reagent (Pierce Biotechnology, Rockford, IL).

Expression Analysis in *P. pastoris*. The recombinant colonies of *P. pastoris* were cultured in YPD at 30 °C and 300 rpm. The culture supernatants were then filtered through 0.22 μm GSWP nitrocellulose membranes (Millipore, Billerica, MA). When necessary, samples were concentrated and dialyzed by employing a 0.01 M potassium phosphate buffer, pH 6, in an Amicon device (Millipore) harboring 10 kDa factor membranes, and finally the enzyme activity was analyzed. In some cases the samples obtained were subjected to an activation treatment carried out with an acidification−neutralization process, as described by Emtage et al. (*10*) or were treated with endoglycosidase H (Sigma-Aldrich, St. Louis, MO) following the manufacturer's instructions. Finally, the enzyme activities of the samples thus treated were analyzed.

To evaluate the enzymatic activity at the intracellular level, 1 mL of lysing buffer (10 mM potassium phosphate buffer pH 6.0, 1% DMSO, 1 mM EDTA, 1 mM PMSF, and 1 μg/mL DTT) and glass beads were used to break prewashed cells obtained from a 25 mL culture. The mixture was centrifuged for 30 min at 45000 rpm and the supernatant used to analyze the enzymatic activity.

Enzymatic Assays. *Fluorescein Thiocarbamoyl-κ-casein Assays.* This assay was carried out to test *k*-caseinolytic activity of the enzymatic

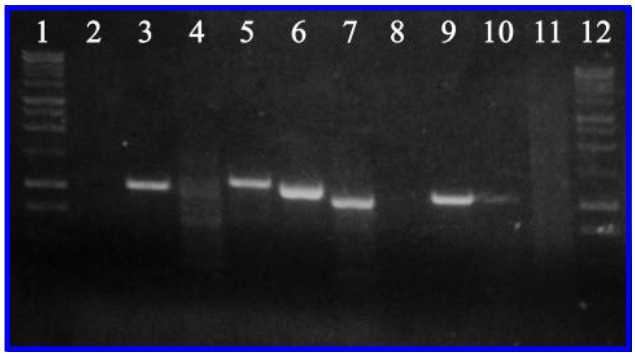

Figure 1. Electrophoresis of RT-PCR products using different oligonucleotide pairs: (lanes 3, 6, and 9) 1101 bp band corresponding to prochymosin; (lane 5) 1146 bp band corresponding to preprochymosin; (lane 7) 975 bp band corresponding to chymosin; (lanes 1 and 12) molecular weight standard 1 kbp ladder (Promega).

samples as described by Ageitos et al. (*20*). One unit of enzymatic activity was defined as the amount of enzyme necessary to degrade 1 μg of FTC-*k*-casein in 1 h at 37 °C. This assay was also employed for the determination of the kinetics parameters and the optimal pH and temperature values.

Milk-Clotting Assay. This assay was performed to test milk clotting, using powdered skimmed milk (Central Lechera Asturiana, Asturias, Spain) reconstituted at 26% (w/v) in 10 mM potassium phosphate buffer, pH 6, as substrate (*21*). The enzyme reactions were carried out in test tubes with 200 μL of enzyme solution and 200 μL of substrate. The milk clots were visualized by turning the tubes upside down. As a negative control, the culture supernatant of a *P. pastoris* clone containing the pGAPZαA vector without any insert was used. As a positive control, commercial rennet of animal origin (Bescansa, Coruña, Spain) was employed. Milk diluted in buffer was used as blank. All assays were done in triplicate.

Finally, 10 mL of supernatant containing active chymosin was employed to elaborate milk curds using 1 L of unpasteurized full-fat cow's milk.

Partial Characterization of the Recombinant Enzyme. *SDS-PAGE.* Polyacrylamide gel electrophoresis was accomplished according to the method of Laemmli (*22*) in a miniProtean-III cell (Bio-Rad, Hercules, CA). The molecular weight of the proteins was determined using Precision Plus Protein Standard (Bio-Rad) and the software Quantity One (Bio-Rad).

Determination of Kinetic Parameters. Michaelis−Menten plots (*23*) were performed with increasing substrate concentrations as described by Ageitos et al. (*20*). The V_{max} and K_m values of the enzymatic samples were obtained using the Lineweaver−Burk method (*23*).

Determination of Optimal Temperature and pH. The enzymatic reactions were subjected to different incubation temperatures (from 10 to 70 °C) over 1 h. They were also carried out at different pH values (from 2.0 to 9.0) for 1 h. The buffers employed were 500 mM sodium citrate (pH 2−3.5), 500 mM sodium acetate (pH 4−5.5), 500 mM potassium phosphate (pH 6−7.5), and 500 mM Tris-HCl (pH 8−9). Enzymatic activity was then measured as described above.

RESULTS

Isolation and Cloning of Preprochymosin, Prochymosin and Chymosin Genes. Using RT-PCR and the primer pairs preprochym and chymosin, prochym and chymosin, and chym and chymosin (**Table 1**), three bands of sizes 1146, 1101, and 975 bp were obtained (**Figure 1**) corresponding to the preprochymosin, prochymosin, and chymosin genes, respectively. Sequencing of the cDNA fragments revealed similarities of 97.9, 97.1, and 96.8%, respectively, with respect to their *B. taurus* counterparts. The three fragments were cloned into the pCR Blunt II TOPO vector, where their DNA sequence was confirmed and submitted to Genbank with accession code EU265816.

Expression of Buffalo Chymosin in *P. pastoris.* Preprochymosin, prochymosin, and chymosin were subcloned into the pGAPZαA expression vector under the control of the pGAP constitutive promoter. The constructions obtained were then integrated into the *P. pastoris* GS115 genome by recombination events. Only the clones containing the prochymosin sequence exhibited active chymosin to the supernatant, without the need of in vitro activation process (**Figure 2b**). The supernatant containing the active buffalo chymosin showed an excellent ability in milk curd elaboration and an optimal whey separation (**Figure 2a**). The production curve made with the strain containing the prochymosin gene showed a maximum of activity after 40 h of incubation, which coincided with the beginning of the stationary phase of growth. This activity was maintained after 100 h of incubation and then started a gentle decrease (**Figure 3**).

The specific activities found at the intracellular level and in the culture supernatant of the strain containing prochymosin are shown in **Table 2**.

Partial Characterization of B. arnee bubalis Chymosin Expressed in P. pastoris. **Figure 4** shows the different protein profiles of supernatant of *P. pastoris* recombinant cultures carrying the pGAPZαA vector with the *B. arnee bubalis* prochymosin gene. In lane 3 (strain producing active chymosin) two bands can be observed of 40 and 36 kDa, respectively, that are not present in lane 2 (negative control), where only the larger one appears. These two bands correspond to the different glycosylation patterns given by *P. pastoris* from *B. arnee bubalis* chymosin, as demonstrated when endoglycosidase H was used, because both bands were fused into one of 36 kDa (lane 4) after enzymatic treatment. The optimum pH of the recombinant chymosin was 4.5, and the enzyme proved to be active over a temperature range of 25−45 °C, its optimum being 37 °C. The K_m value was 0.01223 mM κ-casein and the V_{max} value was 314.165 units/mL.

DISCUSSION

The main objective of the present work was the production of active recombinant chymosin in the culture supernatant of *P. pastoris*. To date, recombinant active chymosin has not been yet obtained in the supernatants of yeasts such as *S. cerevisiae* (*12*) or *K. lactis* (*13, 14*), although it has been obtained previously from supernatants of filamentous fungi such as *T. reesei* (*15*) or *A. oryzae* (*16*). Nevertheless, filamentous fungi are not as good candidates as yeasts for performing large-scale fermentations (*17*).

In this work, preprochymosin, prochymosin,and chymosin genes were isolated from RNA extracted from the abomasum of a male suckling *B. arnee bubalis* calf using RT-PCR techniques. The DNA sequences obtained (Genbank accession code EU265816) showed high homology values with respect to their *B. taurus* counterparts. For the expression of these three sequences in *P. pastoris*, the pGAPZαA integrative vector was used to clone them under the control of the pGAP constitutive promoter and in-frame with the extracellular secretion signal of the *S. cerevisiae* α factor (*24*). Only the cloned prochymosin sequence led to the active chymosin form. This could be explained in terms of *P. pastoris* being unable to appropriately process the signal peptide of 16 amino acids contained in the preprochymosin molecule, which must be removed to generate prochymosin. In the case of the cloned chymosin, this construct did not show any activity,and this could be explained assuming that active chymosin could be generated only when appropriate

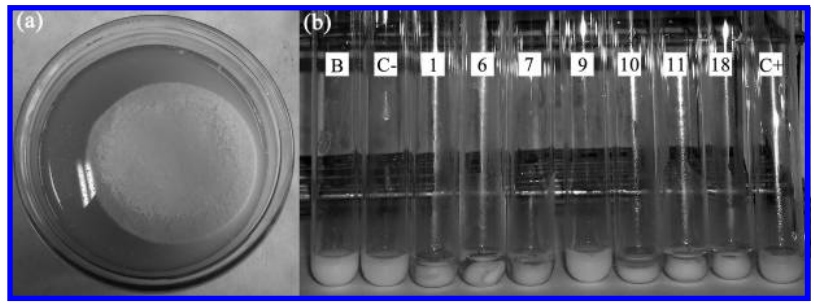

Figure 2. (**a**) Milk curd elaborated with the culture supernatant of a recombinant *P. pastoris* strain containing the prochymosin gene; (**b**) milk coagulation assays carried out with different enzymatic samples obtained from the culture supernatant of a recombinant *P. pastoris* strain containing the prochymosin gene: B, negative control containing milk with 10 mM potassium phosphate buffer pH 6; C, milk treated with the culture supernatant of *P. pastoris* containing the pGAPZαA vector without insert; 1, 6, 7, 9, 10, 11, and 18, milk with the culture supernatants of different recombinant *P. pastoris* clones containing prochymosin; C+, milk treated with commercial animal rennet.

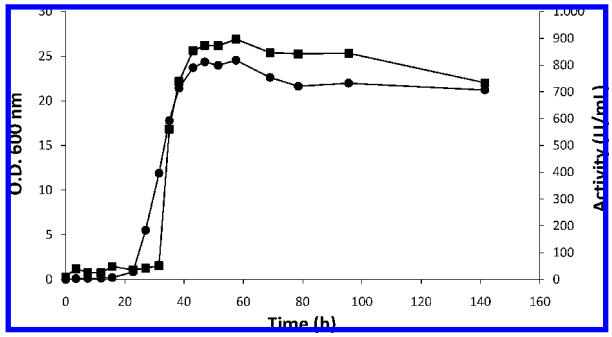

Figure 3. Growth (●) and chymosin acivity (■) curves obtained from the recombinant *P. pastoris* strain containing the prochymosin gene during 140 h.

Table 2. Culture Supernatant and Intracellular Specific Activities of the Active Chymosin from the *Pichia pastoris* Strain Containing the Prochymosin Gene

	supernatant (units/mg of protein)	intracellular (units/mg of protein)
control[a]	1.86 ± 1.43	3.88 ± 0.75
sample[b]	16339.39 ± 1102.74	12.23 ± 0.61

[a] *P. pastoris* strain containing the pGAPZαA vector without insert. [b] *P. pastoris* strain containing the prochymosin gene.

three-dimensional folding occurs in the prochymosin phase. Processing of the α-factor involves several distinct proteolytic cleavage steps, carried out by different enzymes. The 19 amino acid signal peptide (presequence) is removed by a signal peptidase system; further processing of the prosequence then involves the action of an endopeptidase encoded by the KEX2 gene, which cleaves C-terminally to a specific Lys−Arg sequence and a dipeptidyl aminopeptidase encoded by the STE13 gene, which removes N-terminal Glu−Ala repeats (*24*). The amino acidic sequence of the prochymosin contains the target Lys−Arg used by the endopeptidase codified by KEX2 gene. This endopeptidase could cleave this target, generating a protein active in the supernatant similar to that named as pseudochymosin by Pedersen et al. (*15*).

As shown in **Table 2** almost all of the active chymosin is produced in the culture supernatant, and the residual activity values found in the intracellular samples could be due to a small fraction of active enzyme retained at the cell wall level.

In the protein electrophoresis experiments, the supernatant containing the active chymosin originated two bands that did not appear in the control. The larger molecules could be converted into the small one by treatment with endo H, suggesting that some molecules receive at least one *N*-oligosaccharide residue. No changes in mobility were observed

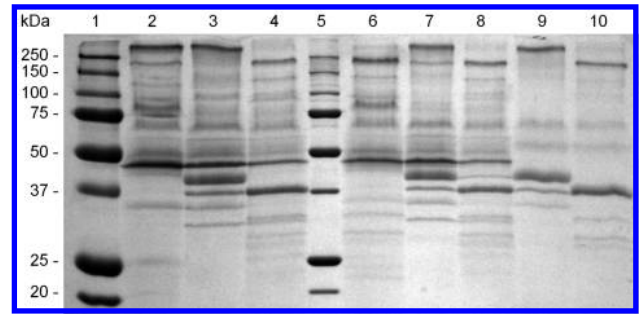

Figure 4. SDS-PAGE carried out with different enzymatic samples obtained from supernatant of the *P. pastoris* strain containing prochymosin: (lanes 1 and 5) molecular weight standards (Precision Plus Protein all blue standards, Bio-Rad); (lanes 2 and 6) negative controls obtained from the culture supernatant of the *P. pastoris* strain containing the pGAPZαA vector without insert; (lanes 3 and 7) culture supernatant of a *P. pastoris* strain containing the pGAPZαA vector with prochymosin; (lanes 4 and 8) culture supernatant of a *P. pastoris* strain containing the pGAPZαA vector with prochymosin treated with endoglycosidase H; (lane 9) culture supernatant of a *P. pastoris* strain containing the pGAPZαA vector with prochymosin treated by the acidification/neutralization process; (lane 10) culture supernatant of a *P. pastoris* strain containing the pGAPZαA vector with prochymosin treated with endoglycosidase H and by the acidification/neutralization process.

when the acidification/neutralization protocol was used, suggesting the absence of acid-sensitive linkages.

The characteristics of the *B. arnee bubalis* chymosin produced in *P. pastoris* were very similar to those described in the enzyme obtained by conventional methods (*25*), although its K_m was lower (*25*), suggesting that the recombinant enzyme has a greater affinity for κ-casein. In this work, we describe for the first time a yeast able to express a chymosin gene in its active form. Additionally, it does not need further in vitro processing to become active. We therefore consider that the recombinant strain could be an excellent candidate for exploitation in the cheese-making industry.

Supporting Information Available: Diagram of preprochymosin. This material is available free of charge via the Internet at http://pubs.acs.org.

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