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Recombinant *Lactococcus lactis* fails to secrete bovine chymosin

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Keywords: *Lactococcus lactis*, heterologous expression, bovine prochymosin, bovine chymosin

Bovine chymosin is an important milk-clotting agent used in the manufacturing of cheeses. Currently, the production of recombinant proteins by genetically modified organisms is widespread, leading to greatly reduced costs. *Lactococcus (L.) lactis*, the model lactic acid bacterium, was considered a good candidate for heterologous chymosin production for the following reasons: (1) it is considered to be a GRAS (generally regarded as safe) microorganism, (2) only one protease is present on its surface, (3) it can secrete proteins of different sizes, and (4) it allows for the direct production of protein in fermented food products. Thus, three genetically modified *L. lactis* strains were constructed to produce and target the three different forms of bovine chymosin, prochymosin B, chymosin A and chymosin B to the extracellular medium. Although all three proteins were stably produced in *L. lactis*, none of the forms were detected in the extracellular medium or showed clotting activity in milk. Our hypothesis is that this secretion deficiency and lack of clotting activity can be explained by the recombinant protein being attached to the cell envelope. Thus, the development of other strategies is necessary to achieve both production and targeting of chymosin in *L. lactis*, which could facilitate the downstream processing and recovery of this industrially important protein.

Introduction

Bovine chymosin (EC 3.4.23.4) is the major enzyme responsible for the coagulation of milk in the abomasum of suckling calves.¹ This aspartyl proteinase is secreted as a 365 amino acid inactive precursor prochymosin, which can be converted into active chymosin in the acidic environment of the stomach by proteolytic removal of a 42 amino acid propeptide at the N-terminus. Chymosin recognizes and partially hydrolyzes k-casein, a protein that consists of a hydrophobic and hydrophilic portion and is located on the surface of caseins that are present in milk. Chymosin releases a hydrophilic k-casein glycopeptide, and the insoluble para-k-casein peptide aggregates with other caseins to form an insoluble clot via a Ca²⁺ dependent process.^{2,3}

Chymosin is one of the most important enzymes in the cheese-making process due to its high specificity for casein.^{4,5} Commercial preparations of calf rennet contain two forms of chymosin, denominated chymosin A and B. Chymosin A slightly exceeds chymosin B in proteolytic activity, whereas chymosin B is more stable at low pH. Chymosin A differs from chymosin B in only one amino acid; the A form has an aspartic acid residue at

position 286, whereas the B form has a glycine residue at this position.⁶

The expansion of cheese production is limited by the reduction of chymosin rennet in livestock slaughter, requiring the development of alternative chymosin sources. Using genetic engineering techniques, recombinant chymosin has been expressed in several microbes, including *Escherichia coli*, *Bacillus subtilis*, and *Kluyveromyces lactis*.^{4,7-9} Microbiologically produced recombinant chymosin costs less and is obtained with high purity and in abundant quantities.¹⁰ Several biotechnology companies produce recombinant chymosin for commercial use in the cheese-making process.⁴ Genetically modified organism (GMO)-based rennet is now used in 80% of global cheese production.¹⁰

Lactic acid bacteria (LAB) are a group of gram-positive, non-sporulating bacteria that produce lactic acid, a common metabolic end product from the fermentation of carbohydrates. LAB are widely used in the food industry for production and preservation of fermented products. These bacteria are also considered to be GRAS (generally regarded as safe) organisms.¹¹ *Lactococcus (L.) lactis*, the model LAB, is a good candidate for heterologous protein production, especially for recombinant protein secretion. Through a Sec-dependant pathway, *L. lactis* is able to

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secrete recombinant proteins of different sizes. Secretion of these proteins leads to the direct production of several proteins in fermented food products facilitating the interaction of the secreted proteins (enzymes or antigens) and their environment (the food products themselves or the digestive tract of animals that consume these engineered bacteria).¹² Due to this, it would be advantageous to use lactococci as chymosin producers because no foreign components of the host would be added to the cheese vat.

For these reasons, genetically modified *L. lactis* strains were constructed for the production and targeting, to the extracellular medium, of three derivative forms of the bovine chymosin gene and evaluated for their milk-clotting potential.

Results

Cloning of synthetic prochymosin B gene using the XIES system

Considering that calf chymosin is an important enzyme involved in the process of milk coagulation and commonly used in cheese manufacturing, the principal aim of the present work was to evaluate the production and secretion of active chymosin by *L. lactis* strains. For this purpose, we constructed three different recombinant *L. lactis* strains using the xylose-inducible expression system (XIES),¹³ which targets the secretion of the protein of interest to the extracellular medium. Three derivatives of the calf chymosin cDNA gene, prochymosin B, chymosin A, and chymosin B, were used to construct the recombinant lactococci strains.

The prochymosin B ORF (*Bos taurus*; NM_180994.1), with all codons optimized for its expression in *L. lactis*, was commercially synthesized and cloned into the pBluescript II SK (+/−) vector resulting in the pBSIISK:prochyB vector (Epoch Biolabs Inc). The prochymosin B gene sequence was used as a template to obtain the chymosin A and B ORFs (Fig. 1). The nucleotide sequences of these genes were cloned into the pXYSEC expression vector after the ribosome binding site (RBS) and the DNA sequence encoding a signal peptide (SP) of the major lactococcal secreted protein Usp45 (RBS_{usp45} and SP_{usp45}). Usp45 is one of the few secreted proteins of *L. lactis* and is the only secreted protein expressed in sufficient quantities to be detected on protein gels stained with Coomassie Blue.¹⁴ The pXYSEC plasmid was successfully used to clone the prochymosin B gene in frame with the signal peptide. To obtain the plasmid that would target the prochymosin protein to the *L. lactis* extracellular medium (pXYSEC:prochyB), the prochymosin gene was PCR amplified from

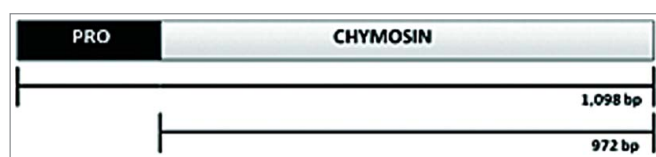


Figure 1. Structure and length of prochymosin and chymosin.

pBSIISK:prochyB (Table 1) using primers *prochyB F* and *prochyB R* (Table 2). The PCR product was digested with the corresponding restriction enzymes, purified and cloned into the purified NsiI-EcoRV-cut pXYSEC expression vector. The construction of the recombinant pXYSEC:prochyB plasmid (Table 1; Fig. 2A) was confirmed via enzymatic digestion and DNA sequencing. The confirmed plasmid was first transformed in *E. coli* TOP10 cells and then transferred into *L. lactis* NCDO2118 (wild type) cells, which were used as the cloning host, resulting in the *LL pXIES:prochyB* recombinant strain. This is the first time that *L. lactis* NCDO2118 has been used to express prochymosin.

L. lactis is able to produce a stable form of synthetic prochymosin B but does not secrete it

To evaluate whether the *LL pXIES:prochyB* strain was able to produce the synthetic prochymosin B and target it outside the cell, western blotting analysis was performed using the proteins extracted from the cell (C) and supernatant (S) fractions of induced and non-induced *LL pXIES:prochyB* and *L. lactis* NCDO2118 cultures. Analysis of induced *LL pXIES:prochyB* samples revealed a 43 kg.mol^{−1} polypeptide in the C fraction, which corresponds to prochymosin B fused to the signal peptide of the Usp45 protein (SP_{Usp45}:prochyB). These polypeptides were not detected in control extracts (Fig. 2B). No proteins were observed in the S fraction (data not shown).

Because the constructed *LL pXIES:prochyB* strain was not able to target the synthetic prochymosin B to the extracellular medium, we decided to investigate the lack of chymosin secretion by *LL pXIES:prochyB*. Therefore, to determine whether the protein was being retained in the cell wall or in the cytoplasm of the bacteria, the induced *L. lactis* NCDO2118 and *LL pXIES:prochyB* cultures were subjected to cell fractionation, and the protein contents in both the cell (C) and cytoplasmic (T) fractions were analyzed using western blot analysis.

High concentrations of prochymosin B were observed in the cell fraction of *LL pXIES:prochyB* induced cultures, and the presence of prochymosin B attached to the cell envelope was evident. This result could be due to the presence of inclusion bodies. In the cytoplasmic fraction, a second band with an approximate molecular weight of 35.6 kg/mol was also observed (Fig. 3), which could represent degradation products. The detected polypeptides were chymosin specific as they were not detected in control *L. lactis* NCDO2118 cells and were recognized by specific anti-chymosin antibodies.

Cloning active chymosin genes using the XIES system

Two new *L. lactis* strains producing the active forms of chymosin A and B were also constructed in an attempt to improve protein secretion efficiency. The ability of *L. lactis* to secrete the different forms of mature chymosin (chymosin A or B) was evaluated. The chymosin B ORF was PCR-amplified from the pBSIISK:prochyB vector using the *chyB F* and *chyB R* primers (Table 2), and the chymosin A ORF was obtained by site-directed mutagenesis of the chymosin B sequence using overlap

Table 1. Bacterial strains and plasmids used in this work

Strain and/or plasmid	Relevant characteristics	Source
Bacterial strains		
<i>E. coli</i> TOP10	(F ⁻ ϕ80dlacZΔM15 Δ(lacZYA-argF)U169 endA1 recA1 hsdR17(r _K -m _K +) deoR thi-1 supE44 λ ⁻ gyrA96 relA1)	Invitrogen™
<i>L. lactis</i> NCDO2118	<i>L. lactis</i> subsp. <i>lactis</i> (vegetable strain, plasmid free)	Collection strain ^a
<i>LL pXIES:prochyB</i>	<i>L. lactis</i> NCDO2118 strain harboring pXYSEC:prochy B	This work
<i>LL pXIES:chyA</i>	<i>L. lactis</i> NCDO2118 strain harboring pXYSEC:chy A	This work
<i>LL pXIES:chyB</i>	<i>L. lactis</i> NCDO2118 strain harboring pXYSEC:chy B	This work
Plasmids		
pBSIISK:prochy B	pBluescript II SK (Amp ^r /pUC ORI) cloning vector carrying prochymosin B gene with lactococcal codon usage	Epoch Biolabs Inc
pXYSEC:Nuc	pWV01/Cm ^r ; expression vector containing the fusion <i>rbS_{Usp45}::sp_{Usp45}::nucB</i> , under the control of P _{xyIT}	Miyoshi and cols., 2004
pXYSEC:prochy B	pXYSEC expression vector carrying prochymosin B gene with lactococcal codon usage	This work
pXYSEC:chy A	pXYSEC expression vector carrying chymosin A gene (obtained by mutagenesis) with lactococcal codon usage	This work
pXYSEC:chy B	pXYSEC expression vector carrying chymosin B gene with lactococcal codon usage	This work

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extension PCR (Fig. 4) with the *chyB* F, *chyB* R, *chyA* mut F, and *chyA* mut R primers (Table 2).

To obtain vectors that were able to target chymosin A and B to the extracellular medium of *L. lactis*, the same procedure adopted to construct pXYSEC:prochyB was performed resulting in the pXYSEC:chyA and pXYSEC:chyB expression vectors. The pXYSEC:chyA (Fig. 5A) and pXYSEC:chyB (Fig. 5B) vectors were constructed by ligating the NsiI-EcoRV-digested pXIES vector with the NsiI-EcoRV-digested chymosin A and B PCR products, respectively. The construction of these expression plasmids was confirmed via enzymatic digestion and DNA sequencing. The plasmids were initially transformed into *E. coli* TOP 10 cells and then into *L. lactis* NCDO2118 cells, resulting in the *LL pXIES:chyA* and *LL pXIES:chyB* recombinant strains.

Chymosin A and B were produced by *L. lactis* but remain in the cells

The recombinant strains (*LL pXIES:chyA* and *LL pXIES:chyB*) were constructed in an attempt to obtain efficiently secreted chymosin in its active form. Once again, as with the *LL pXIES:prochyB* strain, the secretion of the chymosin proteins was not observed.

Proteins extracted from the induced *LL pXIES:chyA* and *LL pXIES:chyB* cultures were analyzed using western blotting to evaluate whether these strains were able to produce active chymosin

and target it to the extracellular medium. Polypeptides of 39 kg.mol⁻¹ were observed in the cell fractions (C), corresponding to mature chymosin A or B fused to SP_{Usp45} (Fig. 5C and D), but no proteins were observed in the supernatant fractions (S). These results showed that direct expression of chymosin A and B by the *L. lactis* XIES system is limited to the cell fraction as no secretion was observed in the extracellular medium.

L. lactis producing chymosin A and B strains do not show clotting activity

Clotting activity was evaluated using solubilized products of the constructed *L. lactis* recombinant strains (*LL pXIES:prochyB*, *LL pXIES:chyA*, and *LL pXIES:chyB*). To develop a simple and sensitive assay to determine milk-clotting activity, a microtiter plate platform was used. No milk-clotting activity was observed in the recombinant strains or in *L. lactis* NCDO2118 (negative

Table 2. Mutagenic and flanking primers used to clone the chymosin genes into expression vectors

Primers	Sequence (5'-3') ^b	Restriction enzyme site
<i>prochyB</i> F	GATGCATACTGAAATTA ^b CTCGAATTC	NsiI
<i>prochyB</i> R	GGATATCTTAGATGGCCTTGGCAAG	EcoRV
<i>chyB</i> F	TCGATGCATCA GGTGAGGTTGCAAGTGTTCCTC	NsiI
<i>chyB</i> R	GGAATTCCTAGATGGCCTTGGCAAGTCC	EcoRI
<i>chyA</i> mut F	GCAACACAAATCAATATGAC ^b gaattgacatagattgtgac	-
<i>chyA</i> mut R	GTCATATTGATTTGTGTTGCTCCAATAG	-

^bNucleotide substitution is shown in boldface; Underlined nucleotides indicate the cloning site.

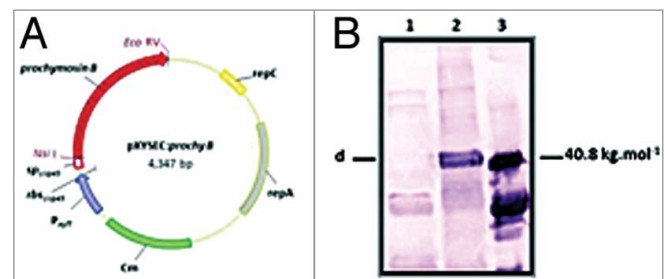


Figure 2. (A) Schematic representation of the plasmid constructed for prochymosin production in *L. lactis*. For details about the plasmid construction, see the text and Table 1. P_{xyIT}: xylose-inducible promoter; RBS_{Usp45}: ribosome binding site of the Usp45 gene; sp_{Usp45}: DNA sequence encoding the signal peptide of the Usp45 gene; prochymosin B: *Bos taurus* precursor chymosin B coding sequence based on lactococcal codon usage. Plasmid constructed for prochymosin production with secreted addressing (not to scale) (B) Immunodetection of recombinant prochymosin produced by *L. lactis*. Analysis of proteins extracted from the cell fraction using polyclonal anti-chymosin antibodies. Lanes 1 and 2: non-induced and induced *LL pXIES:prochyB* cultures, respectively; lane 3: commercial bovine chymosin (Sigma). d: prochymosin fused with the lactococcal Usp45 signal peptide (SP_{Usp45}) (approximately 43 kg.mol⁻¹).

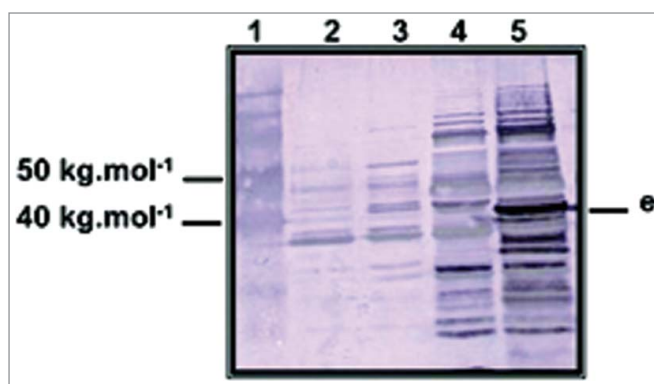


Figure 3. Immunodetection to determine the cellular localization of recombinant prochymosin B using polyclonal anti-chymosin antibodies. Analysis of proteins extracted from the cell (C) and cytoplasmic (T) fractions of the *L. lactis* NCDO2118 (wild type) and *LL pXIES:prochyB* strains from induced culture samples. Lane 1: molecular weight standard (BenchMark Protein Ladder; Invitrogen); lanes 2 and 3: T and C protein fractions of *L. lactis* NCDO2118, respectively; lanes 4 and 5: T and C protein fractions of *LL pXIES:prochyB*, respectively. e: prochymosin fused with the signal peptide of lactococcal Usp45 (SP_{usp45}) (approximately 43 kg.mol⁻¹).

control), even after increasing the incubation time of the cultures from 15 min to 2 h (data not shown).

Discussion

Genetically modified microorganisms have been produced that can yield large quantities of pure chymosin (identical to animal chymosin) at very low cost. In this work, we proposed the use of the *L. lactis* NCDO2118 strain, which can easily express and control heterologous protein secretion using the XIES system, as a new alternative for the production of chymosin as it represents a safe strategy in cheese manufacturing. The XIES expression system represents an interesting genetic tool for obtaining biomolecules with pharmaceutical, medical or industrial relevance.^{15,16}

Here, for the first time, three different strains of *L. lactis* were constructed for the production of the different forms of chymosin, prochymosin B (precursor form) and chymosin A and B (active forms). These results show that the three chymosin forms can be expressed normally by the *L. lactis* XIES system but cannot be secreted. They remain stacked in the cell fraction and present no biological activity.

The *LL pXIES:prochyB* strain clearly produced the chymosin precursor, which was detected in the cell envelope. No secretion or clotting activity was observed using this strain. Similar results were obtained with the *L. lactis* strains that produced chymosin A and B (*LL pXIES:chyA* and *LL pXIES:chyB*, respectively). Considering that protein secretion in *L. lactis* occurs through a Sec-dependent pathway, which has been used to produce and successfully secrete different heterologous proteins, our results suggest that the transport of prochymosin B and chymosin A and B, via

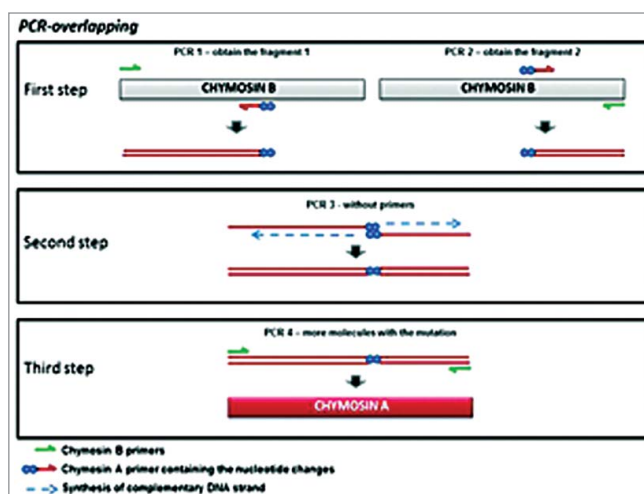


Figure 4. Schematic representation of the overlap extension PCR method used.

this Sec pathway, could be impaired. It is known that recombinant expression can be limited in *L. lactis* because expression of the target proteins can be subject to inadequate stability and/or solubility, leading to improper protein folding, inclusion body formation, protein degradation^{17,18} and/or can be related to the inefficiency of the secretion process.⁸ Thus, it is possible that a post-translational process, such as insoluble aggregate formation, could be limiting chymosin secretion. This represents a bottleneck in the production of soluble proteins that are intrinsically associated with bacterial cell machinery conditions.

The secretion of prochymosin is important for obtaining the activated protein; nevertheless, it seems that recombinant production of this precursor is limited by its failure to form disulfide bonds characterizing the formation of an insoluble cytoplasmic prochymosin. Prochymosin is an extremely hydrophobic protein that has been produced in *E. coli*, *B. subtilis* and yeast (cytoplasmic production) in its insoluble form, and, to become active, the protein had to undergo a denaturing and refolding process.^{7,8} The molecular alteration of prochymosin is not able to significantly change its hydrophobic nature because even deletion of more than 70% of the protein-coding sequence was unable to prevent significant accumulation of the insoluble protein.¹⁹ An alternative to overcoming prochymosin aggregation and to enhance its solubility, biological activity, and secretion is to fuse the prochymosin B coding sequence to other DNA sequences.²⁰ In *Proteus mirabilis*, the coding sequence was fused to exotoxin type A streptococcal pyrogenic (Sep-A) with the promoter, RBS, and signal peptide of Sep-A. Bovine prochymosin was secreted into the extracellular medium, converted to its active form by autocatalytic process and was capable of coagulating milk.²¹ *L. lactis* strains harboring plasmids with different fusion cassettes for expression and/or secretion of prochymosin and proteinase-prochymosin fusion proteins produced intra and extracellular prochymosin. The ratio of secreted to intracellular prochymosin increased with increasing length of the fusion cassette. This

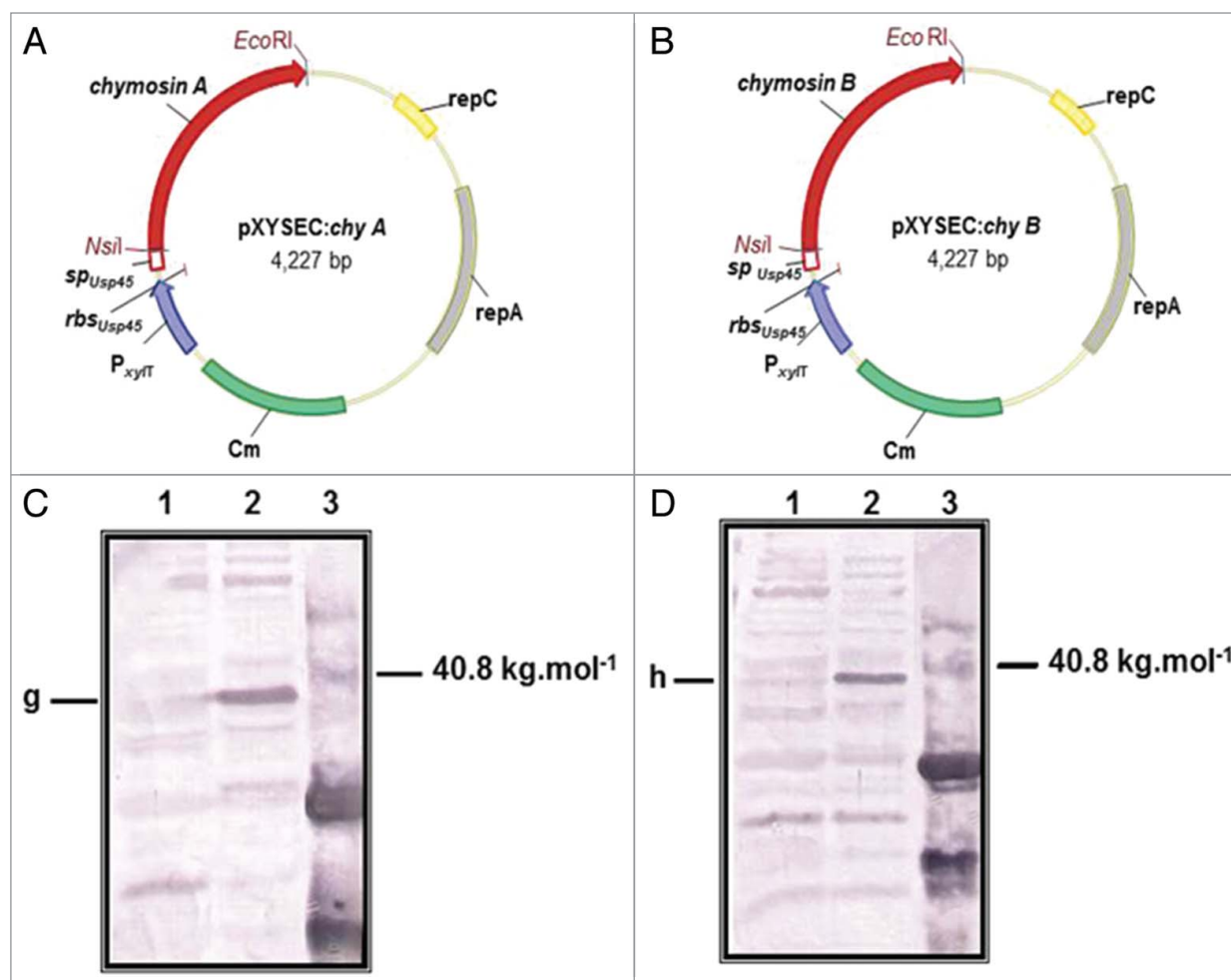


Figure 5. (A, B) Schematic representation of the plasmids constructed for chymosin A and B production in *L. lactis*. For details about plasmid construction, see the text and **Table 1**. P_{xylT} : xylose-inducible promoter; rbs_{Usp45} : ribosome binding site of the Usp45 gene; sp_{Usp45} : DNA sequence encoding the signal peptide of Usp45 gene; chymosin A: *Bos taurus* active chymosin A coding sequence obtained by site-directed mutagenesis of chymosin B; chymosin B: *Bos taurus* active chymosin B coding sequence based on lactococcal codon usage (A) plasmid constructed for chymosin A production with secreted addressing. (B) plasmid constructed for chymosin B production with secreted addressing (not to scale) (C, D) Immunodetection of recombinant chymosin A and B produced by *L. lactis* strains. Analysis of proteins extracted from the cell fraction using polyclonal anti-chymosin antibodies. (C) Lanes 1 and 2: *LL pXIES:chyA* non-induced and induced culture samples, respectively; lane 3: commercial bovine chymosin (Sigma). (D) Lanes 1 and 2: *LL pXIES:chyB* non-induced and induced culture samples, respectively; (lane 3) commercial bovine chymosin (Sigma). g and h: chymosin A and B fused with the signal peptide of lactococcal Usp45 (SP_{Usp45}) (approximately 38.7 kg.mol^{-1}), respectively.

represents a promising strategy for use in *L. lactis* because the fusion of the prochymosin sequence (or even chymosin A and B) with leader sequences from other lactococci gene coding sequence, could result in its secretion by *L. lactis*.²² Furthermore, the insertion of a 9-residue synthetic propeptide LEISSTCDA could enhance the efficiency of chymosin secretion by *L. lactis*.¹²

In this work, the mature forms of the chymosin genes were cloned and expressed in *L. lactis* for the first time. The expression of mature chymosin A and B by recombinant *L. lactis* strains (*LL pXIES:chyA* and *LL pXIES:chyB*, respectively) was successfully observed, but secretion and clotting activity were not detected. Few studies describe the cloning and expression of chymosin in its active form. However, this product cannot be easily obtained, as was shown in a *B. subtilis* clone that was genetically modified to produce mature chymosin. Despite confirmation of the clones,

they were not able to produce the protein.⁸ The inefficiency in producing mature chymosin and presenting its milk-clotting activity has been observed in recombinant *Saccharomyces cerevisiae* strains. Furthermore, it has also been observed that some chymosin polypeptides are associated with the cell wall, and because no chymosin polypeptides are detected in the soluble fractions, this suggested that the synthesized protein could be rapidly associated with cell wall debris or was in an insoluble form.²³ In *E. coli*, direct expression of the chymosin gene would be expected to lead to production of chymosin containing an additional methionine residue at the NH2 terminus which can cause deleterious effects on the production of active chymosin. There was also the possibility that an NH2-terminal methionine would modify the activity or tertiary structure of the enzyme, proving that active chymosin expression in *E. coli* presented disadvantages.⁷

Even after protein solubilization, the recombinant *L. lactis* strains lacked clotting activity. It is possible that the low production of heterologous protein could be responsible for the lack of clotting activity. To improve the production of the heterologous protein, different versions of chymosin were cloned into plasmids with different characteristics (promoter, RBS, ATG, fusion protein). Low production of prochymosin in *B. subtilis* was significantly increased (10×) by coupling the *lpp* gene coding sequence.^{8,24} The protein synthesis efficiency is based on a two-sequences arrangement and it relies on translational coupling for which the second prokaryotic ribosome-binding site sequence, RBS, is dependent of the first sequence upstream of it.⁸

The improvement of expression systems has facilitated obtaining recombinant chymosin mostly from genetically modified fungi. *Pichia pastors* has been successfully used to produce chymosin from different sources using different promoters and expression systems.²⁵⁻²⁷

Considering these results, new expression system tools are being developed to improve the secretion of chymosin by the *L. lactis* NCDO2118 strain.

Conclusions

This work is part of an ongoing project that aims to produce enzymes or antigens in *L. lactis* that are beneficial to animal and/or human health. Here, we have described the construction of recombinant lactococcal strains that produce three derivatives of calf chymosin, the most important enzyme in cheese production with high specificity for k-casein.²⁸

The three constructed recombinant strains were able to produce a stable form of chymosin but were unable to export it to the extracellular environment. The failure of secretion and the presence of proteins stacked in the cellular fraction is most likely the result of an alteration in protein folding, which could improve production, secretion and translocation processes. Thus, the development of other strategies, which could facilitate the downstream processing and recovery of this protein, will be necessary to achieve both production and targeting of chymosin in *L. lactis*.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

Bacterial strains and plasmids used in this study are listed in **Table 1**. *Escherichia* (*E.*) *coli* TOP10 cells were aerobically grown in Luria-Bertani medium at 37°C with agitation. *L. lactis* NCDO2118 cells were grown in M17 medium supplemented with 0.5% (w/v) glucose (GM17) or 1% (w/v) xylose (XM17; when induced) at 30°C without agitation. When required, media were supplemented with chloramphenicol (Cm; 10 µg mL⁻¹).

DNA manipulations

Unless otherwise indicated, DNA manipulation techniques were performed according to standard protocols. Plasmid DNA

was isolated from *E. coli* and *L. lactis*, as previously described.^{29,30} PCR amplifications were performed using Platinum Pfx DNA Polymerase (InvitrogenTM). DNA fragments were isolated from the agarose gels and purified using the *GFXTM PCR DNA and Gel Band Purification* system (GE Healthcare). DNA restriction and modification enzymes were used as recommended by the suppliers. The integrity of all cloned ORFs was confirmed by sequencing performed on double-stranded plasmid DNA using the dideoxy chain termination method.³¹

Construction of the synthetic prochymosin B ORF

The prochymosin B ORF (*Bos taurus*; NM_180994.1) was synthesized with all codons optimized for expression in *L. lactis*^{32,33} and cloned into the pBluescript II SK (+/–) vector by Epoch Biolabs Inc. The resulting vector (*pBSIISK:prochyB*), harboring the synthetic prochymosin B, was used as a template to obtain the chymosin A and B ORFs (**Fig. 1**).

Primer design

All primers used in this work were designed using the Vector NTI 10 software (Invitrogen) and are shown in **Table 2**. The primers were designed based on the synthetic prochymosin B and contained the appropriate restriction sites for subsequent cloning steps.

Isolation of the prochymosin B, chymosin A, and chymosin B ORFs

The prochymosin B and chymosin B ORFs were PCR-amplified from the pBSIISK:prochyB vector using the *prochyB F* and *prochyB R* or *chyB F* and *chyB R* primers, respectively.

The chymosin A ORF was obtained by site-directed mutagenesis of the chymosin B coding sequence (**Fig. 4**). For site-directed mutagenesis, four primers were used: (1) a pair of primers flanking the chymosin B ORF (*chyB F* and *chyB R*) and (2) a pair of mutagenic primers with an average 21-bp overlap between adjacent fragments (*chyA mut F* and *chyA mut R*). The mutation site was located in these mutagenic primer sequences.

Site-directed mutagenesis was performed using the overlap extension-PCR method that consists of a three-step PCR procedure.³⁴ Briefly, two simultaneous PCR reactions were performed in the first step. One reaction was performed with the *chyB F* and *chyA mut R* primers and the other reaction was performed with the *chyA mut F* and *chyB R* primers.

To obtain the full-length mutated fragment coding chymosin A, the products from both PCR reactions were mixed (approximately 60 ng each) and used as templates for a subsequent overlap extension-PCR reaction. This PCR was performed without adding primers and using the same conditions previously described. A final PCR reaction was performed using the full-length DNA product previously obtained and the pair of chymosin B primers (*chyB F* and *chyB R*). The amplified products were gel purified and digested with *NsiI* and *EcoRI* restriction enzymes (**Table 2**) before being cloned into the pXYSEC expression vector.

Construction of *L. lactis* strains targeting prochymosin B, chymosin A and chymosin B to the extracellular medium

The PCR fragment containing the prochymosin B ORF was purified, digested with *Nsi*I and *Eco*RV and cloned directly into the *Nsi*I and *Eco*RV-digested pXYSEC expression vector, resulting in the pXYSEC:prochyB vector. The same procedure was adopted to clone the chymosin A and B ORFs, resulting in the pXYSEC:chyA and pXYSEC:chyB vectors, respectively. The vectors were first transformed into *E. coli* TOP10 cells and then transferred after purification to *L. lactis* NCDO2118 cells by electroporation. The final lactococcal strains were as follows: (i) *L. lactis* (pXYSEC:prochyB), hereafter called *LL*(pXYSEC:prochyB); (ii) *L. lactis* (pXYSEC:chyA), hereafter called *LL*(pXYSEC:chyA); and (iii) *L. lactis* (pXYSEC:chyB), hereafter called *LL*(pXYSEC:chyB).

Xylose induction conditions

The wild type *L. lactis* NCDO2118 strains or the recombinant strains (harboring pXYSEC:prochyB, pXYSEC:chyA or pXYSEC:chyB) were grown overnight in 5 mL of GM17 media. The overnight culture was diluted 1:10.000 in 10 mL of fresh XM17 media to induce the expression of the prochymosin and chymosins ORFs. Incubation at 30°C was continued until the cultures reached an optical density of 2.0 at 600 nm (OD₆₀₀), before performing protein extractions.

Protein extraction

Protein extractions of the supernatant, cell, and cytoplasmic fractions were performed as previously described.¹⁷ Briefly, 2 mL of exponential-phase cultures [optical density at 600 nm (OD₆₀₀), 1.5 to 2.0] was centrifuged at 4°C for 10 min at 17,500 × g. The supernatants and the cell pellets were processed separately. The supernatants were filtered through 0.2 μm filters (low protein retention; Millipore) to remove the bacteria and proteins from 1.5 mL of the filtrate were precipitated with 100 μL of ice-cold 100% (w/v) trichloroacetic acid. The mixture was kept on ice for 60 min and then centrifuged at 4°C for 15 min at 17 500 × g. The resulting pellet was dissolved in 75 μL per OD₆₀₀ unit of the 50 × 10⁻³ mol L⁻¹ of NaOH. The cell fraction was resuspended in 100 μL of TE-Lys buffer (1 × 10⁻³ mol L⁻¹ EDTA, 50 × 10⁻³ mol L⁻¹ TRIS-HCl [pH 7.5], lysozyme [10 mg mL⁻¹]), homogenized, incubated in a water bath for 30 min at 37°C and then 33 μL of 20% (w/v) sodium dodecyl sulfate (SDS) per OD₆₀₀ unit of the original culture was added.

Proteins of the cytoplasmic fraction were obtained from the pellet and treated with TE-Lys solution in the same manner as the cell fraction. However, the samples were centrifuged for 10 min at 17 500 × g at 4°C. The supernatants were filtered (0.22 μm) and underwent the same treatment as the supernatant fraction, described above.

References

1. Foltmann B. A review on prorennin and rennin. C R Trav Lab Carlsberg 1966; 35:143-231; PMID:5330666
2. Crabbe M. Rennets: General and Molecular Aspects Amsterdam: Elsevier Ltd., 2004.
3. Creamer LK, Plowman JE, Liddell MJ, Smith MH, Hill JP. Micelle stability: kappa-casein structure and function. J Dairy Sci 1998; 81:3004-12; PMID:9839241; [http://dx.doi.org/10.3168/jds.S0022-0302\(98\)75864-3](http://dx.doi.org/10.3168/jds.S0022-0302(98)75864-3)
4. Mohanty AK, Mukhopadhyay UK, Grover S, Batish VK. Bovine chymosin: production by rDNA technology and application in cheese manufacture. Biotechnol Adv 1999; 17:205-17; PMID:14538140; [http://dx.doi.org/10.1016/S0734-9750\(99\)00010-5](http://dx.doi.org/10.1016/S0734-9750(99)00010-5)

Polyclonal anti-chymosin production

BALB/c mice (5 to 6 wk old) were obtained from the Federal University of Minas Gerais (UFMG, Brazil). The animals were immunized with 20 μg of purified chymosin (Sigma). Three doses were administered subcutaneously at intervals of 15 d. Freund's complete adjuvant was administered with the first dose, and Freund's incomplete adjuvant was administered with subsequent doses. Samples were collected on days 0, 14, 28, and 42 after the first dose. The first samples were taken from the retro-orbital plexus, and the last samples were taken from the heart. The humoral immune response was evaluated by an indirect ELISA assay (enzyme-linked immunosorbent assay).

Western blot analysis

Equal volumes of 2X loading buffer were added to all protein samples. Protein extracts were subjected to SDS-PAGE (12% acrylamide) and western blotting using anti-chymosin antibodies, as previously described.²¹ Immunodetection was performed using *Western Breeze*[®] Chromogenic Western Blot Immunodetection (Invitrogen), as recommended by the manufacturers.

Chymosin milk-clotting activity assay

The three recombinant *L. lactis* strains, producing prochymosin B, chymosin A or chymosin B, were analyzed for clotting activity. Prochymosin was converted to catalytically active chymosin by acidification and/or neutralization (activation) immediately prior to the assay, first by acidification to pH 2.0 for 15 min at room temperature and then by adjusting the pH to 6.3; incubation was continued for 1 h. Non-induced strains and the *L. lactis* NCDO2118 wild type strain were used as negative controls.

For solubilization of aggregate chymosin, we used the Marston et al. (1984) protocol.³⁵ The extract was concentrated using Amicon[®] Ultra (10.000 MW cut-off) centrifugal filters. Twenty-five microliters of each sample were used to evaluate chymosin milk-clotting activity using a previously described technique by Emtage et al. (1983), with incubation times of 15 min or 2 h.⁷

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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5. Møller KK, Rattray FP, Sørensen JC, Ardö Y. Comparison of the hydrolysis of bovine κ -casein by camel and bovine chymosin: a kinetic and specificity study. *J Agric Food Chem* 2012; 60:5454-60; PMID:22563811; <http://dx.doi.org/10.1021/jf300557d>
6. Rampilli SB, Molinari P, Tenaglia L. HPLC analysis of rennet enzymes and fermentation-produced chymosin. *Scienza e Tecnica Lattiero Casearia* 1992; 43:387-401
7. Emtage JS, Angal S, Doel MT, Harris TJ, Jenkins B, Lilley G, Lowe PA. Synthesis of calf prochymosin (prorennin) in *Escherichia coli*. *Proc Natl Acad Sci U S A* 1983; 80:3671-5; PMID:6304731; <http://dx.doi.org/10.1073/pnas.80.12.3671>
8. Parente D, de Ferra F, Galli G, Grandi G. Prochymosin expression in *Bacillus subtilis*. *FEMS Microbiol Lett* 1991; 61:243-9; PMID:1903749; <http://dx.doi.org/10.1111/j.1574-6968.1991.tb04356.x>
9. van den Berg JA, van der Laken KJ, van Ooyen AJ, Renniers TC, Rietveld K, Schaap A, Brake AJ, Bishop RJ, Schultz K, Moyer D, et al. *Kluyveromyces* as a host for heterologous gene expression: expression and secretion of prochymosin. *Biotechnology (N Y)* 1990; 8:135-9; PMID:1366557; <http://dx.doi.org/10.1038/nbt0290-135>
10. Johnson ME, Lucey JA. Major technological advances and trends in cheese. *J Dairy Sci* 2006; 89:1174-8; PMID:16537950; [http://dx.doi.org/10.3168/jds.S0022-0302\(06\)72186-5](http://dx.doi.org/10.3168/jds.S0022-0302(06)72186-5)
11. Miyoshi A, Bermudez-Humaran LB, Azevedo MSP, Langella P, Azevedo V. Lactic Acid Bacteria as Live Vectors: Heterologous Protein Production and Delivery Systems in *Biotechnol. Lact. Acid Bact.* Oxford: Wiley-Blackwell, 2010.
12. Langella P, Le Loir Y. Heterologous protein secretion in *Lactococcus lactis*: a novel antigen delivery system. *Braz J Med Biol Res* 1999; 32:191-8; PMID:10347754; <http://dx.doi.org/10.1590/S0100-879x1999000200007>
13. Miyoshi A, Jamet E, Commissaire J, Renault P, Langella P, Azevedo V. A xylose-inducible expression system for *Lactococcus lactis*. *FEMS Microbiol Lett* 2004; 239:205-12; PMID:15476967; <http://dx.doi.org/10.1016/j.femsle.2004.08.018>
14. van Asseldonk M, Rutten G, Oteman M, Siezen RJ, de Vos WM, Simons G. Cloning of *usp45*, a gene encoding a secreted protein from *Lactococcus lactis* subsp. *lactis* MG1363. *Gene* 1990; 95:155-60; PMID:2123812; [http://dx.doi.org/10.1016/0378-1119\(90\)90428-T](http://dx.doi.org/10.1016/0378-1119(90)90428-T)
15. de Azevedo MS, Rocha CS, Electo N, Pontes DS, Molletta JB, Gonçalves ED, Azevedo V, Silva CL, Miyoshi A. Cytoplasmic and extracellular expression of pharmaceutical-grade mycobacterial 65-kDa heat shock protein in *Lactococcus lactis*. *Genet Mol Res* 2012; 11:1146-57; PMID:22614283; <http://dx.doi.org/10.4238/2012.April.27.14>
16. Miyoshi A, Jamet E, Commissaire J, Renault P, Langella P, Azevedo V. A xylose-inducible expression system for *Lactococcus lactis*. *FEMS Microbiol Lett* 2004; 239:205-12.
17. Le Loir Y, Gruss A, Ehrlich SD, Langella P. A nine-residue synthetic propeptide enhances secretion efficiency of heterologous proteins in *Lactococcus lactis*. *J Bacteriol* 1998; 180:1895-903; PMID:9537390
18. Le Loir Y, Azevedo V, Oliveira SC, Freitas DA, Miyoshi A, Bermúdez-Humarán LG, Nouaille S, Ribeiro LA, Leclercq S, Gabriel JE, et al. Protein secretion in *Lactococcus lactis*: an efficient way to increase the overall heterologous protein production. *Microb Cell Fact* 2005; 4:2; PMID:15631634; <http://dx.doi.org/10.1186/1475-2859-4-2>
19. McCaman MT. Fragments of prochymosin produced in *Escherichia coli* form insoluble inclusion bodies. *J Bacteriol* 1989; 171:1225-7; PMID:2536676
20. Ward M, Wilson LJ, Kodama KH, Rey MW, Berka RM. Improved production of chymosin in *Aspergillus* by expression as a glucoamylase-chymosin fusion. *Biotechnology (N Y)* 1990; 8:435-40; PMID:1366537; <http://dx.doi.org/10.1038/nbt0590-435>
21. Klessen C, Schmidt KH, Gumpert J, Grosse HH, Malke H. Complete secretion of activable bovine prochymosin by genetically engineered L forms of *Proteus mirabilis*. *Appl Environ Microbiol* 1989; 55:1009-15; PMID:2499253
22. Simons G, Rutten G, Hornes M, Nijhuis M, van Asseldonk M. in *Struct. Funct. Aspartic Proteinases* (Springer US) 1991; 306:115-9; http://dx.doi.org/10.1007/978-1-4684-6012-4_14
23. Mellor J, Dobson MJ, Roberts NA, Tuite MF, Emtage JS, White S, Lowe PA, Patel T, Kingsman AJ, Kingsman SM. Efficient synthesis of enzymatically active calf chymosin in *Saccharomyces cerevisiae*. *Gene* 1983; 24:1-14; PMID:6313478; [http://dx.doi.org/10.1016/0378-1119\(83\)90126-9](http://dx.doi.org/10.1016/0378-1119(83)90126-9)
24. Schoner BE, Belagaje RM, Schoner RG. Translation of a synthetic two-cistron mRNA in *Escherichia coli*. *Proc Natl Acad Sci U S A* 1986; 83:8506-10; PMID:3534891; <http://dx.doi.org/10.1073/pnas.83.22.8506>
25. Vallejo JA, Ageitos JM, Poza M, Villa TG. Cloning and expression of buffalo active chymosin in *Pichia pastoris*. *J Agric Food Chem* 2008; 56:10606-10; PMID:18975968; <http://dx.doi.org/10.1021/jf802339e>
26. Jiang XP, Yin ML, Chen P, Yang Q. Constitutive expression, purification and characterization of bovine prochymosin in *Pichia pastoris* GS115. *World J Microbiol Biotechnol* 2012; 28:2087-93; PMID:22806030; <http://dx.doi.org/10.1007/s11274-012-1012-7>
27. Nosedá DG, Recupero MN, Blasco M, Ortiz GE, Galvagno MA. Cloning, expression and optimized production in a bioreactor of bovine chymosin B in *Pichia (Komagataella) pastoris* under AOX1 promoter. *Protein Expr Purif* 2013; 92:235-44; PMID:24141135; <http://dx.doi.org/10.1016/j.pep.2013.08.018>
28. Jacob M, Schmidt M, Jaros D, Rohm H. Measurement of milk clotting activity by rotational viscometry. *J Dairy Res* 2011; 78:191-5; PMID:21411032; <http://dx.doi.org/10.1017/S0022029911000112>
29. Sambrook J. *Molecular Cloning: A Laboratory Manual*. New York: Cold Spring Harbor, 2001.
30. Langella P, Le Loir Y, Ehrlich SD, Gruss A. Efficient plasmid mobilization by pHP501 in *Lactococcus lactis* subsp. *lactis*. *J Bacteriol* 1993; 175:5806-13; PMID:8376328
31. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* 1977; 74:5463-7; PMID:271968; <http://dx.doi.org/10.1073/pnas.74.12.5463>
32. Menzella HG. Comparison of two codon optimization strategies to enhance recombinant protein production in *Escherichia coli*. *Microb Cell Fact* 2011; 10:15; PMID:21371320; <http://dx.doi.org/10.1186/1475-2859-10-15>
33. Villalobos A, Ness JE, Gustafsson C, Minshull J, Govindarajan S. Gene Designer: a synthetic biology tool for constructing artificial DNA segments. *BMC Bioinformatics* 2006; 7:285; PMID:16756672; <http://dx.doi.org/10.1186/1471-2105-7-285>
34. Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 1989; 77:51-9; PMID:2744487; [http://dx.doi.org/10.1016/0378-1119\(89\)90358-2](http://dx.doi.org/10.1016/0378-1119(89)90358-2)
35. Marston FAO, Lowe PA, Doel MT, Schoemaker JM, White S, Angal S. Purification of Calf Prochymosin (Prorennin) Synthesized in *Escherichia Coli*. *Nat Biotechnol* 1984; 2:800-4; <http://dx.doi.org/10.1038/nbt0984-800>