Contents lists available AInCEJ

Angolan Industry and Chemical Engineering Journal

Journal visit: aincej.com

Development of Biotechnological Techniques for the Processing and Use of Bovine Chymosin on an Industrial Scale.

[1]Paulina Gonçalves, [2]Paz García, [3]Gloria Fernandez-Lorente, [4]Miguel Ladero and [3,5]Benevides Pessela

[1]Centro Nacional de Investigação Científica. Av. Ho Chi Min, Nº 201, Cx. Postal 34, Bairro Maianga, Luanda-Angola
[2]Universidad Autónoma de Madrid (UAM). Espanha.
[3]Departamento de Biotecnologia e Microbiologia de Alimentos (CIAL) C/ Nicolás Cabrera 9, 28049, Cantoblanco, Campus UAM. Madrid Espanha.
[4]Departamento de Engenharia Química, Faculdade de Ciências Complutense Av. Séneca, 2, 28040 Madrid, Espanha.
[5]Departamento de Engenharia e Tecnologia. Instituto Superior Politécnico de Tecnologias e Ciências, ISPTEC. Av Luanda Sul S10, Talatona, Luanda-Angola.

ARTICLE INFO

Keywords:
bovine chymosin,
extraction,
coagulation,
magnetic particles.

Chymosin is an enzyme, traditionally obtained from the fourth stomach of young ruminants, used in the coagulation of milk to make cheese. In the present work we will address other aspects that are difficult to resolve during the processing of this protein, its activation, purification process and alternatively, its immobilization in various chromatographic matrices that allow its manipulation under more technically industrial and economically beneficial industrial conditions. The process consisted of cloning the coding gene for this enzyme, using optimized codons for its overexpression in Escherichia coli, with the pBAD/HIS vector under the control of the pBAD promoter inducible by L-arabinose, as it is a more economically profitable process. The process consisted of cloning the coding gene for this enzyme, using optimized codons for its overexpression in Escherichia coli, with the pBAD/HIS vector under the control of the pBAD promoter inducible by L-arabinose, as it is a more economically profitable process. Overexpression of the gene generates inclusion bodies that can be denaturalized by washing with NaOH, for subsequent opening of the protein by dilution and adjustment of the pH with glycine, in a more easily manipulated way. Once unfolded, prochymosin is activated by a drastic change in pH, first at pH 2 and then rising to pH5 to increase its activation process. After this process, a highly active protein was obtained, tested under its natural substrate, milk. Due to the complexity of the activation-reactivation- purification process that takes place at very acidic pHs, its control becomes difficult, which is why new alternatives are being sought to overcome this inconvenience. The more conservative alternative found was the use of immobilization techniques on different polymeric supports (agarose based) and on magnetic particles (magnetic nanoparticles), previously functionalized, so that in a single step we could carry out the purification process and protein activation. When using polymeric supports, the immobilization results were very poor (below 20%), mainly due to pore clogging problems in this type of resin; whilst, the use of magnetic nanoparticles completely changed the scenario, obtaining yields of almost 100% and retention of enzymatic activity at values close to 85%.

1. Introduction

Proteases (also called peptidases) are enzymes that break the peptide bonds of proteins. They are synthesized and found naturally in living beings, and are involved in the digestion of proteins, facilitating their degradation, absorption and metabolism [1]. Proteases are one of the most important types of enzymes in terms of industrial application. Thus, it is used in the detergent, food, leather processing and pharmaceutical industries [2-4].

Studies have described that they are found in animals, plants, bacteria, fungi, retroviruses and viruses where they perform various functions, from the digestion of proteins in the digestive system of vertebrates to the breakdown of viral polyproteins essential for their multiplication [5]. The aspartic proteins, as the main characteristic of aspartic acid residues, have a bilobed three-dimensional conformation, where the active site is found in both lobes, characteristics that give them the ability to act specifically on the Phe105- Met106 link present in the casein of it milk, originating the coagulation of this [6].

Chymosin is secreted in the fourth stomach of newborn calves and other young ruminants. [7-9]. Chymosin is synthesized intracellularly, in the form of pre- and pro-chymosin, characterized as a protein with 381 amino acid residues. The initial 16 hydrophobic amino acid residues represent the signal peptide or pre-peptide, important in the excretion of the enzyme across the cell membrane. This signal peptide is removed during excretion and in the stomach the enzyme is present in the inactive form, prochymosin or pro-peptide, with a molecular mass of 40,777 Daltons. Under acidic stomach conditions, at pH less than 5, prochymosin is converted to chymosin by catalytic activation and loses 42 N- terminal amino acid residues [10,11]. Chymosin is an extremely important enzyme for cheese production; However, the growing demand for this enzyme in the cheese production market has been reduced in recent years because producers have not been able to guarantee its demand in the industry, which makes it necessary to search for alternative sources to obtain it [12].

Ethical factors also influence the search, as to obtain the enzyme it is necessary to sacrifice the animal, and the yield achieved is quite low. It is also reflected in communities with specific religious norms or vegetarian/vegan eating habits [3]. After several years of research related to this topic, as a substitute for animal chymosin, with the increase in demand for this enzyme, the scientific community developed cheaper biotechnological alternatives with higher yields in large-scale production. One of the most successful alternatives has to do with recombinant DNA techniques using genetic engineering methodologies. There are several genetic constructs for the expression of animal chymosin in a wide variety of microorganisms, among which we find *Escherichia coli*, *Pichia pastoris* and *Saccharomyses cerevisiae*. According to [13], the *E. coli* expression system was the first to be used to produce recombinant pharmaceutical products and presents some advantages in relation to

eukaryotic systems. *Escherichia coli* was one of the most used microorganisms for the production of heterologous proteins, according to [14], due to its rapid growth in low-price culture media, its easy genetic manipulation and the large number of commercial vectors and expression systems are available to scientists, are the main advantages of using these microorganisms. And the disadvantage of the same system is the high level of expression, so sometimes proteins tend to aggregate into inclusion bodies, and generally require long renaturation and folding processes to become functional again [14-15].

Protein purification comprises a series of processes that allow a single type of protein to be isolated from a complex mixture. Protein purification is of great importance to specify the function, structure and interactions of the protein of interest. Typically, the material

used is a microbial culture or biological tissue. To carry out the purification process, there are different steps such as: freeing the protein from the matrix that limits it, separating the protein and non-protein parts of the mixture and, subsequently, separating the expected protein from everything else [16].

Research on enzyme immobilization is a simple technique and allows the beneficial management of enzyme preparations, which is why it has generated great interest in the scientific community [17]. Immobilization aims to explore the economics of biocatalytic processes, as the technique allows operational stability, viability and greater functional efficiency of the enzyme, reuse of the enzyme, improved reproducibility of results, minimum reaction time and simpler separation of the catalyst of the product [18, 19]. In general, enzyme immobilization methods are classified as physical and chemical. Differentiating into: physical, the interactions between the enzyme and the support are weak, and while the chemicals, the enzyme and the support are joined through covalent bonds [20].

According to the type of bond involved in solid supports or in the matrix, there are different immobilization strategies that involve: adsorption, covalent bonding, encapsulation, entrapment in organic and inorganic matrices and copolymerization in polysaccharides such as chitosan, anionic polysaccharides, oligosaccharide derivatives. (polyglucuronic acid), etc. [21].

The fact that proteases (chymosin) are capable of self-lysing, which leads to their faster inactivation, has motivated the search for immobilization and stabilization strategies on a suitable support, thus favoring their refolding. An ideal material as a support for immobilization must have high affinity for proteins, availability of reactive functional groups to react with enzymatic groups or susceptible to chemical modifications, easy preparation in different physical forms, be non-toxic and have physiological compatibility, etc. For the immobilization of bovine chymosin, it was decided to use Agarose supports with different sizes and activated with different groups such as: epoxides, IDA (Iminodiacetic acid linked to metal chelates, cationic and anionic supports (DEAE, sulfopropyl) and finally magnetic nanoparticles materials considered almost ideal for this type of reaction due to their nature.

Currently, there has been increasing interest in the use of enzymes immobilized in magnetic nanoparticles, as it is a choice that allows their recovery through an external magnetic field, thus prolonging successive application cycles and useful life. It is a new process, currently used for a wide range of applications, but to date there is little history of immobilization of these enzymes and their application. [21]. Magnetic nanoparticles are defined as structures that comprise particles, with dimensions between 1 and 100 nm and that may contain special characteristics; they are also capable of functioning as support for other molecules or being directed in a system [21]. Various compositions of magnetic nanoparticles can be synthesized, such as: iron oxides (Fe_2O_3 and Fe_3O_4); cobalt, manganese, nickel and magnesium ferrites, FePt, γ-Fe_2O_3, cobalt, iron, nickel, α- Fe, CoPt and FeCo [22]. The processes for synthesizing magnetic nanoparticles can be by coprecipitation, thermal decomposition, hydrothermal synthesis and using microemulsion for the synthesis of high-quality nanoparticles [21].

Due to all these characteristics mentioned above, magnetic nanoparticles used as support in immobilization have several biotechnological applications in different areas such as: biomedicine, diagnosis, environmental biotechnology, energy, and food sector, analysis of biomarkers in virus detection and in the development of enzymatic biosensors [19].

2. Materials and methods

2.1. Materials

Starting gene, pPFZ- R2 (American Association of Type Crops (ATCC); Strains DH10B, BL21 (DE3); vectors, pBAD/His and PET 29 (a+) ATG biosynthics GmbH; Culture media, Luria-Bertani (LB) , Terrif Broth (TB), LB- Agar; Skimmed milk powder (Sveltesse); Commercial Chymosin Comes from Proquimica SA Coruã- Galicia, Recombinant Chymosin produced at CIAL; Agarose 4BCL; DEAE-agarose, Sulphopropyl sepharose fast Flow,CNBR-Sepharose -4BCL; Hieral(II) sulfate, Ammonium Oxalate, Hieral(III) sulfate, Ammonium hydroxide 30%, Sodium dodecyl sulfate (SDS), Ammonium oxalate monohydrate, magnetic stirrer, water bath, thermostat, magnetic magnets, nitrogen Sigma/Aldrich.

2.2. Methods

Construction of the synthetic bovine chymosin A gene for expression in Escherichia coli using the pBAD/His and PET 29 (a+) vectors.

a) Crop harvest

The biomass obtained in the fermentation process after centrifugation at 5000 x g for 20 minutes and at a temperature of 4°C. It was resuspended in 20 mL of 1 mM EDTA and centrifuged again for 20 minutes with the same centrifugal force and temperature. The sediment was used at the same time or frozen at -80°C for further processing.

b) Determination of the coagulant activity of chymosin

To determine the coagulant activity of recombinant chymosin, the method based on the calculation of International Milk Coagulation Units (IMCUS) was used according to [20], which refers to the amount of enzyme capable of coagulating 2.5 mL of milk at 10% w/v in one minute at a temperature of 37°C.

2.2.1. Coagulation Assay

2.5 mL of previously prepared milk was used, added to 50 mL bottles and left to preheat in a water bath at 37°C for 10 - 15 min maximum. Then, 250 µl of the enzyme solution was added and the time was measured with a stopwatch. Keeping the bottle in a water bath, it was manually shaken every 30 seconds. The clotting time was defined precisely when the first milk clots appeared on the walls of the bottle. This time was considered the clotting time. The tests were carried out in triplicate, always using commercial chymosin as a positive control, following the same procedure, but adding 3.5 10 µ.

2.2.2. Biochemical Characterization

Determination of the effect of pH and temperature, stability against pH and temperature, on the derivative immobilized on magnetic nanoparticles of the commercial and recombinant enzyme.

To evaluate the influence of the pH effect of the enzymes, they were evaluated in the range of 5.0 to 7.5 using 20 mM acetate as a buffer for the pH range 5.0-5.5- and 20-mM Tris-HCL for the pH range 6-7.5. To evaluate the effect of temperature, the reaction was carried out at different incubation temperatures: 20, 25, 30, 37, 40, 45, 50, 60°C respectively.

a) Immobilization

The agarose was prepared and activated and then the activated supports were prepared with Epoxide groups and consequently the IDA (Iminodiacetic Acid) agarose according to specific protocols [21].

a) Immobilization on metallic chelate supports Ag-IDA-Ni and Ag-IDA-Cu

Once the agarose-epoxide support was obtained, it was incubated with iminodiacetic acid, the diols generated spontaneously in the support were oxidized. Then, it was washed with plenty of distilled water and treated with a solution of 0.5 g of copper sulfate, dissolved in 100 mL of 50 mM phosphate buffer pH6 and 0.1 g of NaCl, and to this solution 10 g of agarose - epoxide. IDA, left stirring for 2 hours at 25°C. The support was then washed with plenty of distilled water. To synthesize the Ag – IDA-Ni support, the same procedure was followed, adding NiCl2. Then, all these supports were used to immobilize the chymosin.

2.2.3. Synthesis of Magnetic Nanoparticles

Magnetic nanoparticles were produced using the method developed by [22]. 11 g of ferrous sulfate II were added to 5.65 g of ammonium oxalate and dissolved in 100 mL of distilled water, mixed and heated until complete dissolution. Next, 16 g of iron III sulfate and 11 g of iron II sulfate were add. 30% ammonium hydroxide was added to this suspension to correct the pH to 9. Then, the suspension was placed in the reactor, a stream of nitrogen was introduced and stirred vigorously at 1200 rpm for one hour in a water bath at 75ºC. °C. Subsequently, the supernatant was separated from the particles with the aid of a magnetic magnet and the particles were washed with distilled water. Then, about 3 g of SDS was added to the nanoparticle suspension and it was adjusted to pH 5.0. The Fe_3O_4: SDS molar ratio is 2:1. ed to the suspension. Thus, the dicarboxylic groups that are on the surface of the particles were replaced by sulfate groups. The solution continued to operate under the same working conditions for 24 hours. Finally, the particles were washed with 5 portions of hot (deoxygenated) water at 60 to 65°C. Decanting was done with the aid of a magnet. The solution obtained was frozen for 24 hours and then lyophilized until a completely dry sample was obtained. For immobilization, the following were used as support: 0.5g of nanoparticles, 1 ml of enzyme, 4 ml of buffer, and the same procedure was carried out as the immobilization process.

3. Results and Discussion

3.1. Heterologous Expression System for the Bovine Chymosin Enzyme

With the strain acquired from ATCC, producer of the commercial chymosin pPFZ-R2, with the aim of purifying the plasmids and manufacturing a new synthetic gene to produce recombinant chymosin protein. Once the strains were cultivated, we performed DNA electrophoresis in 0.7% agarose and the result appears in Figure 3.

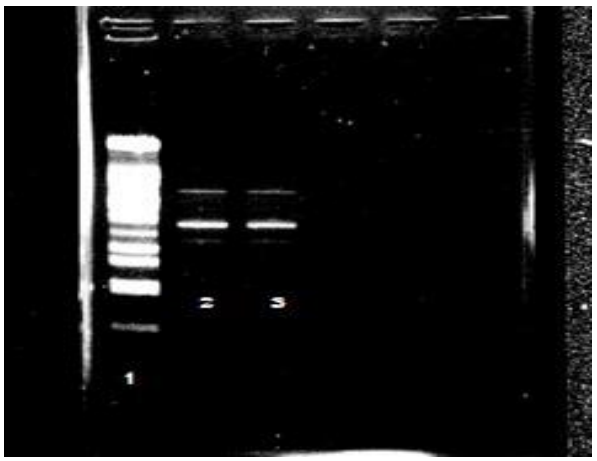

Figure 1*: Electrophoresis in 0.7% agarose. (Lane 1. Molecular weight standards. Lane 2: plasmid extracted from the strain acquired at ATCC (pPFZ-R2) and purified. Lane 3: plasmid extracted from the strain acquired at ATCC (pPFZ-R2) and purified).*

After this experiment, the pPFZ-R2 strain was seeded on an LB-Agarose plate to then isolate individual strains and see their phenotype in the production of chymosin protein. The result of this experiment is presented in Figure 2. In it we can observe that all colonies have the same profile, therefore we can conclude that the culture and strains were completely free of any contamination (Figure 1).

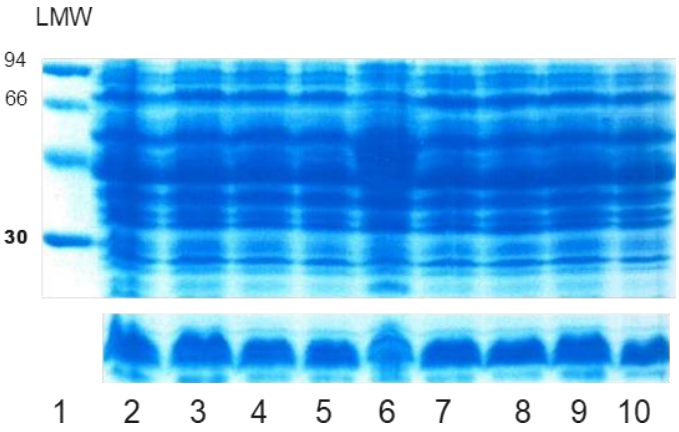

Figure 2*: 12% SDS-PAGE of small cultures of strain pPFZ-R2 under various culture conditions. Lane 1: Low molecular weight standards. Lane 2: colony 1 of pPFZ-R2. No ampicillin. Lane 3: colony 2 of pPFZ-R2. No ampicillin. Lane 4: colony 3 of pPFZ-R2. No ampicillin. Lane 5: colony 4 of pPFZ-R2. Without Ampicillin. Lane 6: colony 2 of pPFZ-R2 with Ampicillin. Lane 7: colony 5 of pPFZ-R2. With ampicillin. Lane 8: colony 6 of pPFZ-R2. With ampicillin. Lane 9: colony 7 of pPFZ-R2. With ampicillin. Lane 10: colony 8 of pPFZ-R2. With ampicillin.*

The synthetic gene obtained was cloned into the pBAD/His and PET 29 (a+) vectors and transformed into several E. coli strains, including strain BL21 (D3) and DH10B. They were cultured and induced overnight, and cellular proteins were analyzed using 12% polychrome gel electrophoresis and cell growth was determined. The cultivation conditions were studied: Arabinose concentration, temperature, stirring speed, carbon and nitrogen source (2%, 37°C, 150 rpm, 0.2% glycerol and 5g/L yeast extract) Once the prochymosin gene induction process was studied and adjusted, optimization of extraction, folding, activation and concentration conditions was addressed. We consider this point to be fundamental since the purification process also takes place.

Once the protein has been activated at pH 2.0, its final activation must be carried out to leave the extract at pH 5.0, typical for its conservation and use. To achieve this, the pH must be raised passing through the isoelectric point of the proteins and this generates certain precipitation and activity problems. In some cases, we are able to actively recover all of the initial enzyme, as we see in the electrophoresis in the figure. In some cases, some yield was lost due to precipitation and because at the isoelectric point both protein forms, pseudochymosin and chymosin, can coexist.

3. Purification of the recombinant enzyme.

There are several methods for enzymatic extraction described in the literature, such as: sonication, enzymatic treatment or pressure rupture. However, in this work we chose to perform the extraction using alkaline treatment due to its operational simplicity and low cost. The use of 0.2 M NaOH, in addition to destabilizing and disrupting cell membranes, also causes complete destabilization of proteins, which is why we were able to separate the chymosin aggregates. So, with simple dilution and adjustment of pH 8.0-9.0 we favor the denaturation of the protein itself since the chymosin molecules are separated enough to fold individually and avoid returning to the state of inclusion bodies. Although there are many methods to dissolve the enzyme to its native conformation, in this work wechose to perform

the denaturation without these (inclusion bodies) due to their complexity and cost.

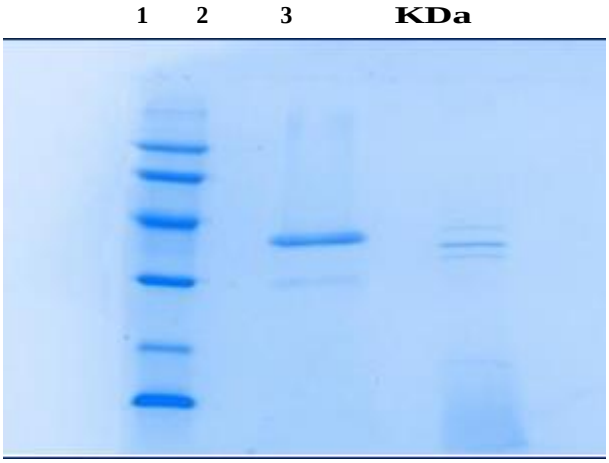

Figure 3: *SDS-PAGE 12%. Complete chymosin activation process. Lane1: Molecular weight standard. Lane 2: Commercial Enzyme. Lane 3: Recombinant Enzyme.*

3.1. Immobilization of enzymes on exchange supports.

By immobilizing the enzymes studied (commercial enzyme and recombinant enzyme) on different supports, derivatives with different activities, stabilities and selectivity were obtained, such as the nanoparticle derivatives for the two enzymes and the metal-IDA-Cu derivative for the enzyme. in study. However, enzymes with sulfopropyl (-), DEAE (+) and metal chelate (Ag-IDA-Ni) supports were difficult to immobilize. Immobilization of commercial and recombinant enzyme preparations on magnetic nanoparticles. For both enzymes, we were able to immobilize 80% of both enzymes on magnetic nanoparticles. The difference in immobilization levels when using other types of supports is due to the clogging effect of the micelles on these supports, which does not happen when using magnetic particles.

Figure 4: *coagulation of 10% skimmed milk and 0.01 M [CaCL2] incubated at 37°C is observed, of the derivative into magnetic nanoparticles using the commercial 1-enzyme and 2-enzyme under study.*

3.1.1. Effect of pH on the derivative immobilized on magnetic nanoparticles commercial and recombinant enzyme).

The difference in immobilization levels when using other types of supports is due to the clogging effect of the micelles on these supports, which does not happen when using magnetic particles. The difference is that from pH 5 the stabilization levels increase in the commercial preparation, but then remain at 100%. On the other hand, the recombinant enzyme is constantly hyperactivated at almost all pH values studied, which constitutes a very striking difference between the two enzymes, Figure 5.

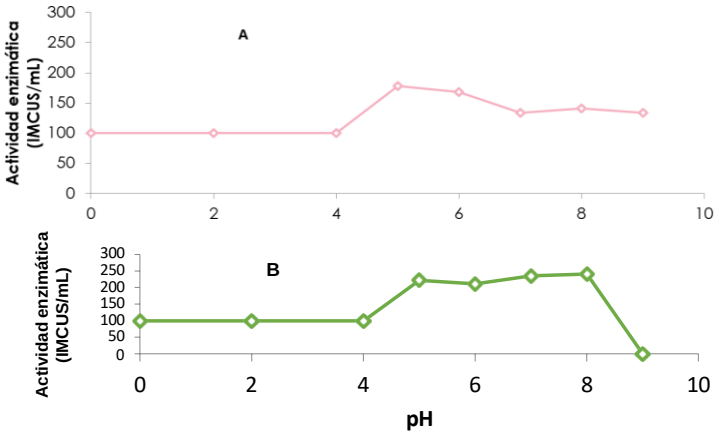

Figure 5: *Effect of pH on coagulation of nanoparticle derivative of commercial enzyme A and recombinant enzyme B.*

This fact is also observed when studying the effect of temperature on activity. Here, both enzymes show similar behavior as constant hyperactivation is observed in both enzymes.

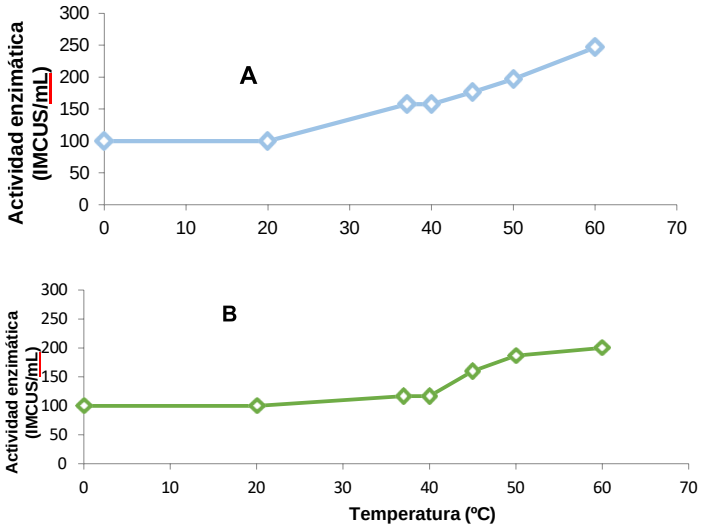

Figure 6: *Effect of temperature on coagulation of nanoparticle derivative of commercial enzyme A and recombinant enzyme B.*

This fact is also observed when studying the effect of temperature on activity. Here, both enzymes show similar behavior as constant hyperactivation is observed in both enzymes.

4. Conclusions

It was possible to develop an effective expression system for the production of bovine chymosin A in E. coli. The main advantage of this expression system is that the promoter used (PBAD) is very closed under normal conditions, making the clones more stable.

The immobilization process on polymeric supports did not give satisfactory results, mainly due to the immobilization processes and the internal morphology of the supports. This fact led to the replacement of this type of supports with magnetic particles, which are more robust and without pore obstruction problems.

The immobilization process appears to be a valuable alternative during complex reactivation processes, as it saves steps normally performed in this process (activation- reactivation) and improves the final performance of the process.

Using polymeric supports, the immobilization results were very poor (below 20%) mainly due to pore obstruction problems in this type of resin; Meanwhile, the use of magnetic nanoparticles has completely

changed the scenario. We were able to immobilize 80% of both enzymes on magnetic nanoparticles. We managed to immobilize 80% of both enzymes on magnetic nanoparticles.

Bibliography

Justesen, SF.; Lamberth, K.; Nielsen, LB.; Schafer-Nielsen, C.; Buus, S., (2009). Recombinant chymosin used for exact and complete removal of a prochymosin derived fusion tag releasing intact native target protein. Protein Sci; 18(5):1023-1032.

Kumar, A.; Grover, S.; Sharma, J.; Batish, V., (2010) Chymosin and other milk coagulants: sources and biotechnological interventions. Crit Rev Biotechnol;30(4):243-258.7

Foltmann, B., (1970). Prochymosin and chymosin. Meth. Ensym.19: 221- 436.

Mohanty, A. K. Mukhapathyay, U. K.; Grover, S.; Batish, V.K., (1999). Bovine Chymosin: Production by rDNA technology an application in cheese manufacture. Biotechnology Advances 17. 205 – 217. Molecular Biology Unit, Dairy Microbiology Division, National Dairy Research Institute, Karnal, 132001.India.

Langholm Jensen, J.; Molgaard, A.; Navarro Poulsen, J. C.; Harboe, M. K.; Simonsen, J. B.; Lorentzen, A. M.; Hjerno, K.; Van Den Brink, J. M.; Qvist,

K. B.; Larsen, S., (2013). Camel and bovine chymosin: the relationship between their Structures and cheese-making properties. Acta Crystallogr D Biol Crystallogr, 69: 901-13.

Morales, J., V.; Muzio, I.C.; Torrens, V.; Jimenez, A.; Silva, A. Santos; Quiñones, Y.; Narciandi, R.E.; Herreira, L.S., (1989). Expresión del Gene de la Quimosina en Escherichia coli. Interferon y Biotecnologia 6:242-250.

Pedersen, V., Christensen, K., Foltmann, B., (1978). Investigations on the activation of bovine prochymosin. Eur. J. Biochem.

Lambertz, C.; Garvey, M.; Klinger, J.; Heesel, D.; Klose, H.; Fischer, R.; Commandeur, U., (2014). Challenges and advances in the heterologous expression of cellulolytic enzymes: a review. Biotechnology for biofuels, v. 7, n. 135, p. 1 – 15.

Choi, J. H.; Keum, K. C; Lee, S.Y., 2006.Production of recombinant proteins by high cell density culture of Escherichia coli. Chemical Engineering Science 61:876 - 85.Adrio, J. L.; Demain, A. L., (2010). Recombinant organisms for production of industrial products. Bioengineered Bugs, v. 1, p. 116–131.

Voet, D., Voet, J.G., (2013). Bioquímica. 4º Edição. Porto Alegre: Artmed,1482p. ISBN: 978-85-8271-004-3.

Arroyo, M., (1990). Inmovilización de enzimas. Fundamentos métodos y aplicaciones. Departamento de bioquímica y biología molecular I. Facultad de ciencias biológicas. Universidad Complutense de Madrid.

Sahu, A.; Badhr, P.S.; Adivarekar, R.; Aniruddha, M.R.L.; Pandit, A. B., (2016). Synthesis of glicinamides using protease immobilized magnetic nanoparticles. Biotechnology Reports. Journal homepage: http://www Elsevier.com/locate//btre.

Yazid, N. A.; Barreira, R.; Sanchez, A., (2017). The immobilization of protease produced by SSF onto functionalized magnetic nanoparticles: Application in the hydrolysis of different protein sources. Journal homepage: http://www Elsevier.com/locate//moleca

Tichy, P. J.; Kapralek, F.; Jecmen, P., (1993). Improved procedure for a high- yield recovery of enzymatically active recombinant calf chymosin from Escherichia coli inclusion bodies. Protein Expr Purif, 4: 59-63.

Mati – Baouche, N.; Elchinger, P. H.; DE-Baynast, H.; Pierre, G.; Delattre, C.; Michaud, P., (2014). Chitosan as an adhesive, Eur, Polm. J. 198 - 213.

Elchinger, P. H.; Delattre, C.; Faure, S.; Roy, O.; Badel, S.; Bernardi, T.; Taillefumier, C.; Michaud, P., (2015). Immobilization of protease on chitosan for the devopement of films with anti – biofim properties, Int. J. Biol. Macromol. 72. 1063 -1058.

Sánchez-Ramírez, J; Martínez-Hernández, J.L.; Segura-Cenice, E. P.; Contreras-Esquivel, J. C.; Medina-Morales, M. A.; Aguilar, C. N.; Iliná, A., (2014). Inmovilización de enzimas lignocelulolíticas en nanopartículas magnéticas. Facultad de Ciencias Químicas, Universidad Autónoma de Coahuila, Blvd. V. Carranza e Ing. José Cárdenas V. Col. República Oriente,

C.P. 25000 Saltillo, Coahuila, México.

Rojas, H.A.; Martínez, J.J.; Vargas H.Y., (2014). Magnetic supports selection for Urease immobilization. Escuela de Ciencias Químicas. Facultad de Ciencias. Grupo de Catálisis (GC-UPTC). Universidad Pedagógica y Tecnológica de Colombia, Avenida Central del Norte, Vía Paipa, Tunja, Boyacá – Colombia. Ingeniería y Competitividad, Volumen 16, No. 2 p. 289 – 296.

International IDF Standard 157 A, 1997 (IMCUS).

Mateo, C.; Abian, O.; Bernedo, M.; Cuenca, E.; Fuentes, M.; Fernandez- Lorente, G.; Palomo, J. M.; Grazu, V.; Pessela, B. C. C.; Giacomini, C.; Irazoqui, G.; Villarino, A.; Ovsejevi, K., Francisco-Vieira, F.; Fernandez – Lafuente, R.; Guisan, J. M., (2005). Some special features of glyoxyl supports to immobilize proteins. Enzyme and Microbial Technology 37. 456 - 462.

Lee, D.G.; Ponvel, K.M.; Kwang, S.; Ahn, L. S. ; Lee,G. H., (2009).

Immobilization of lipase on hidrophobic nano sised magnetite particles. J. Mpl. 66.

Htt://dxdoi.org/10.1016/j.moleatb.2008.06.017.