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Original Article

THE OBTAINING OF THE RECOMBINANT CAMEL CHYMOSIN BY SUBMERGE FERMENTATION IN THE PILOT BIOREACTOR

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ABSTRACT

In the cheese making industry, chymosin is used as a milk-clotting enzyme. With its high specific activity against κ-casein, chymosin better than other proteolytic enzymes. Bactrian camel chymosin has a milk-clotting activity higher than calf chymosin. A scheme for obtaining a milk-clotting preparation based on recombinant camel chymosin is proposed. Submerge fermentation of recombinant yeast *Pichia pastoris* was carried out in a 50-liter bioreactor and recombinant camel chymosin was obtained. The activity of chymosin in the yeast culture was 174.5 U/mL. Chymosin was concentrated 5.6-fold by cross-flow ultrafiltration with 10 kDa cut-off membrane, and chymosin was purified by ion exchange chromatography. The activity of purified chymosin was 4700 U/mL. By sublimation drying with casein peptone, the powder chymosin was obtained with an activity of 36,000 U/g. The proposed scheme for obtaining a milk-clotting drug based on recombinant camel chymosin using submerge fermentation of recombinant yeast has the prospect of being used at biotechnological enterprises.

Keywords: Chymosin, Fermentation, Camel, Calf, Bioreactor, Freeze-drying.

INTRODUCTION

Chymosin (EC 3.4.23.4) also known as rennin is an aspartic protease produced by abomassum glandular cells of newborn mammals [1]. This enzyme specifically break down the Phe105-Met106 peptide bond of milk kappa-casein to for insoluble para-κ-casein [2]. Because of the high specific milk-clotting activity, chymosin is considered the most efficient milk coagulant in the cheese making industry. Natural chymosin isolation from the stomachs of newborn animals is limited by ethical issues and has high economic costs. Simultaneously, the annual increase in cheese production raises the demand for this enzyme. Chymosin can, however, be obtained through recombinant technology [2] in microorganisms [3, 4] and plants [5, 6]. Chymosin expression in fungi and yeast is very efficient because fermentation in bioreactors allows for large amounts of enzyme to be produced in a short period of time [7].

Calf chymosin was the first recombinant enzyme which was approved by FDA [3]. The fermentation-produced chymosin accounted for more than 90% of rennet utilized, and it has the added benefit of being both kosher and halal acceptable [4]. In addition to chymosin of calf, goat and sheep origin, chymosin of camel origin is promising, which is active against cow [5-8], camel [8, 9] and mare [10] milk. In comparison, camel chymosin has higher thermostability relative to bovine chymosin and possesses higher milk-clotting activity [11, 12], making it useful and attractive for commercial cheese manufacturing.

The aim of the work is to obtain recombinant chymosin from the bactrian camel *Camelus bactrianus* under conditions close to industrial conditions. Previous studies have shown high biochemical activity of *Camelus bactrianus* recombinant chymosin, which predisposes to the effectiveness of this chymosin as a milk-clotting enzyme [10]. To continue the work and reach industrial conditions, the following tasks were set: fermentation of the producer strain in a pilot bioreactor, isolation and purification of chymosin, obtaining the powder form of chymosin, and testing the coagulation properties of the enzyme.

The yeast chosen was *Pichia pastoris*, which suitable as a host organism for heterologous expression of proteins owing to simplicity of its genetic manipulation, rapid growth on an inexpensive medium to high cell densities, and capability for complex post-translational modifications [13, 14]. The *Pichia pastoris* yeast expression system is a good choice for obtaining a heterologous recombinant protein with prospects for large-scale microbial production [15].

MATERIALS AND METHODS

Yeast recombinant strain, mediums and chemicals

The recombinant yeast strain - *Pichia pastoris* GS115/pGAPZαA/ProchymCB was used for the production of camel chymosin. The host strain is the methylotrophic yeast *Pichia pastoris*, which harbors the constitutive pGAPZαA vector for chymosin production. Chymosin is expressed constitutively and exported to the broth supernatant and does not precipitate. For fermentation used YEPD medium (1% yeast extract, 2% peptone, 3% dextrose, pH 7.0) and YCB medium (1% yeast extract, 2% peptone, 3% dextrose, 100 mM citrate-phosphate buffer pH 4.0, 10 mM ascorbic acid, 5% sorbitol, 10 g/L beet molasses). Food grade dextrose was purchased from Roquette (France). The peptone and yeast extract were purchased from Titan (India). The chemical reagents used in this study were of molecular biology or pure analytical grade and purchased from Sigma-Aldrich (St. Louis, USA) and AppliChem (Darmstadt, Germany).

Fermentation conditions

50 L pilot fermenter (BioTech 50, Chine) was utilized to determine the ability of the yeast strain to produce *C. bac-*

trianus recombinant chymosin on a large scale. The *Pichia pastoris* GS115/pGAPZαA/ProchymCB strain cells were inoculated into a 50 mL flask containing 5 mL of YEPD with zeocin (100 μg/mL) and were cultured overnight at 30°C and 250 rpm in a shaking incubator (KS 4000i control, IKA, Germany). The overnight culture was inoculated into 50 mL of YEPD in a 500 mL shake flask and was incubated at 30°C and 250 rpm for overnight. The culture was inoculated into 200 mL of the YCB broth and grown at 28°C and 250 rpm for 24 h. Then, the culture was inoculated into 2 L of the fresh YCB broth in the two 5 L flasks. Culture was grown to the OD_{600} = 15-25, and then inoculated in 50 L pilot fermenter in 15L volume. Standard procedures were utilized to operate the fermenter under the following cultivation conditions: 28°C, 100-250 rpm, aeration 120 - 240 L/h, DO – 20-30%, pH 4.0, fed carbon sources: 0.8% glucose and 5% 100 mM citrate-phosphate buffer (pH 4.0) (added each 24 h), and cultivation duration: 144 h.

Harvest cells and culture clarification

The yeast culture was cooled to 6°C by chiller Naser Industrial Chiller (China). Cell pellet was harvested with tubular centrifuge Fuji Separators WGQ-75 (China) at 18000 × rpm. The yeast culture was fed into centrifuge by peristaltic pump SG600FC (China) with 1.5 L/min. The pellet was weighed and clarified culture was stored at 4°C.

The clarified yeast culture was sterilized by microfiltration with bench filtration apparatus UPIRO-018 (Vladisart). The membrane polyethersulfone module MKM46020 (Vladisart) with cut off 0.22 μm and 0.1 m^2 square was used for filtration. Flow rate was 80 mL/min, the temperature 8-10°C. The pH and temperature were monitored for all process.

Activation of chymosin and concentration

The pH of sterile culture pH was decreased to 3.0 with concentrate HCl (10.6 M) at 100 × rpm. The culture incubated at 22°C for overnight and the milk-clotting activity was measured.

The sterile and activated culture was concentrated by cross-flow ultrafiltration on the membrane polyethersulfone module MKM46010 (Vladisart, Russia) with cut off 10 kDa and 0.1 m^2 square. Flow rate was 25 mL/min, temperature 18-20°C. The pH, temperature and enzymatic activity were monitored for all process.

Chymosin purification

Ion exchange chromatography was used to purify recombinant camel chymosin from yeast culture [16]. Concentrated culture supernatant of GS115/pGAPZαA/ProchymCB culture was passed through a 0.22 μm filter and pH was changed to 4.5 with 25 mM sodium acetate, followed by incubation at room temperature for 24 h with stirring to activate all produced chymosin. Activated culture supernatant was diluted 3 times with 25 mM sodium acetate, and pH was lowered to 3.0 with 1M HCl to make chymosin net charge positive. The mixture was loaded onto anion-exchange column with DEAE-Sepharose FF (GE Healthcare, USA) equilibrated with 25 mM NaCl in 50 mM sodium citrate buffer (pH 3.0); this step was defined as clarification. The flow through the column was collected and loaded onto cation-exchange column with SP-Sepharose FF quilibrated with 25 mM NaCl in 50 mM sodium citrate buffer (pH 3.0). The column was next washed with 500 mL of 25 mM NaCl in 50 mM sodium citrate buffer (pH 3.0) and the chymosin was eluted by pH shift with 200 mL of 50 mM NaCl in 25 mM sodium acetate buffer (pH 5.5) to change the net charge of chymosin to negative, and was collected by 40 mL fractions; this step was defined as capture. The fractions with milk-clotting activity were combined, and the concentration of NaCl was lowered to 25 mM by dilution with 25 mM sodium acetate buffer (pH 5.5) and loaded onto the strong cation-exchange column with Q-Sepharose FF (Sigma-Aldrich, USA) equilibrated with 25 mM NaCl in 25 mM sodium acetate buffer (pH 5.5). The column was washed with 25 mM NaCl in 25 mM sodium acetate buffer (pH 5.5), and chymosin was eluted by means of a 50–2000 mM gradient of NaCl in 25 mM sodium acetate buffer (pH 5.5); this step was defined as polishing. The fractions were analyzed by the milk-clotting assay and the most active fractions were applied to SDS-PAGE. The SDS-PAGE was conducted according to the Laemmli method [17] in a Mini-PROTEAN Tetra cell (Bio-Rad Laboratories Inc., USA).

Casein peptone preparation

Casein peptone was made by hydrolyzing skim curd with pepsin. Pepsin was used to coagulate the skim milk into curd (Titan, India). The curd (900 g) was rinsed with distillate water and incubated for 3 hours at room temperature in 450 mL of 90% ethanol. Then, it was air-dried for 16 hours at room temperature. 113 grams of dried casein were ground into a powder, 600 milliliters of distillate water were added, and the combination was incubated for one hour at 80°C in a water bath. The mixture was cooled, the pH was decreased to 2.0 with HCl and the pepsin (1 g) was added for hydrolysis. The hydrolysis was carried out at 45°C for 48 h. In the process of hydrolysis, the pH was kept within 2.0–2.2. After hydrolysis the pH was increased to 4.0 and the hydrolysate was incubated at 80°C for 1 h. The mixture was cooled and pH was increased to 8.0. Non-hydrolyzed casein was precipitated by centrifugation at 10,000 × g, for 1 h and at 4°C. The supernatant was autoclaved and clarified by centrifugation at 10,000 × g, for 1 h and at 4°C. Clarified supernatant was frozen at -80°C in U570 Ultra low freezer (New Brunswick Scientific, USA) and freez-dried in BETA 2-8 LDplus (Christ, Germany) at -90°C under vacuum (0.030 mBar) for 48 h and the dry casein peptone was powdered.

Freeze-drying of the chymosin

The powder chymosin was prepared by freeze-drying of the purified chymosin. The casein peptone (6 g) was added to 88 mL of liquid chymosin. The solution was mixed and spreaded on sterile polyester plate and had frozen at -20°C for 18 h. After that, the plate with frozen chymosin was cooled at -80°C in U570 Ultra low freezer (New Brunswick Scientific, USA) for 3 h. The it was transferred into BETA 2-8 LDplus (Christ, Germany) and was dried under vacuum (0.030 mBar) for 48h. The temperature in condenser was -90°C, the temperature in frozen chamber was -50°C. The lyophilized chymosin was powdered, the moisture was measured on Infrared Moisture Determination Balance MD83 (VIBRA, Shinko Denshi Co., LTD) and the milk-clotting activity of the powder was determined.

Milk-clotting assay

The milk-clotting assay was carried out in accordance

with ref. [18]. This assay was performed with powdered cow's skim milk reconstituted at 12% (w/v) in 0.025 M sodium acetate buffer, pH 6.0, as a substrate. The enzymatic reactions for clone selection were carried out at least in triplicate at 37°C in test tubes with 20 µL of an enzyme solution and 1 mL of the substrate. The milk clots were visualized by turning the tubes upside down. One unit of milk-clotting activity was defined as the quantity of the enzyme required for clotting 1 mL of cow's skim milk in 40 min at 35°C. Activity units of chymosin (A) were calculated by following formula [19]:

$$A = \frac{V_{milk}}{V_{chymosin}} \times \frac{2400}{T_{mc}}$$

there V_{milk} is milk volume (mL), $V_{chymosin}$ is volume of added chymosin (mL), T_{mc} is milk-clotting time (sec).

Determination of protein concentration

Protein concentration was determined by the Bradford method [20] with bovine serum albumin as the standard. Briefly, we mixed 100 µL of the Bradford reagent (Protein Assay Dye; Bio-Rad, Munich, Germany) and 860 µL of 10% PBS with 1% of glycerol and added 40 µL of a protein sample. The mixture was incubated for 2 min at room temperature (RT), and optical density was measured on a spectrophotometer at 595 nm. The measurements were performed on three independent replicates, and the average of the three samples is reported.

RESULTS AND DISCUSSION

Pichia pastoris has become a popular host for the expression and mass production of industrial enzymes [22, 23]. Traditionally *Pichia pastoris* cultivation is performed in fed-batch fermentation using the methanol-inducible system, an AOX1-based expression system. The AOX1 promoter is a strong promoter that can achieve a high level of foreign protein [24]. In this system, excessive accumulation of methanol suppressed cell growth, making process very difficult [25]. Another strong expression system, pGAPZ – based system, is reported to produce protein at a comparative level to the AOX1 – based system, although the level appeared to vary depending on the protein being expressed and the carbon source used for cell growth [26]. For the pGAPZ – based system, foreign protein was expressed constitutively without induction using methanol, which is costly and hazardous to handle in large volume [27].

In our case, the prochymosin gene from *Camelus bactrianus* was inserted under the GAP promoter. The codon optimization of the gene made it possible to obtain a high level of expression. The composition of the medium was chosen successfully. Previously, we have determined that the addition of 10% molasses, 5% sorbitol and 10 mM ascorbic acid to the YEPD medium and pH stabilization with 100 mM citrate buffer increases the yield of recombinant chymosin [10].

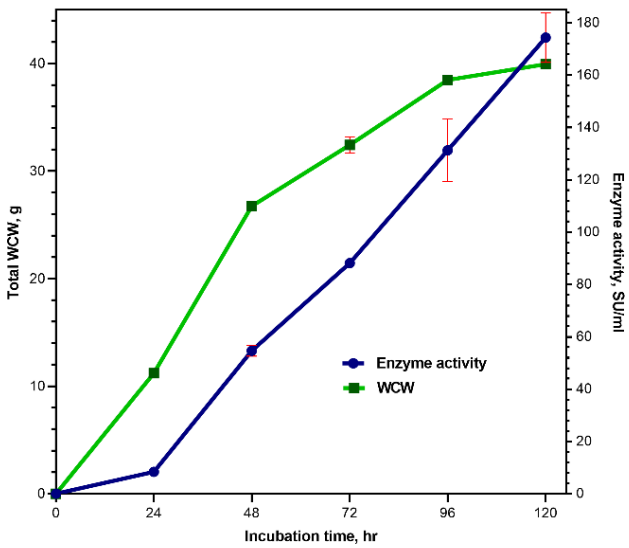

Figure 2. Production of recombinant camel chymosin by yeast *P. pastoris* in 50 L bioreactor

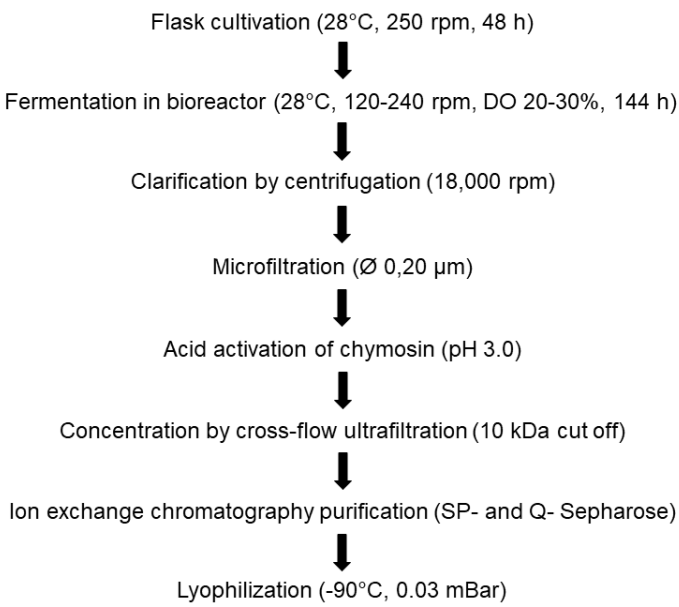

Figure 1. Scheme for obtaining recombinant camel chymosin

The daily addition of 0.8 % glucose as a carbon source required a high oxygen consumption, due to the high metabolism of *Pichia pastoris*, but also allowed to reach 27.8 grams of wet cells per liter of culture. Dissolved oxygen level is critical in aerobic cultivations, especially in the case of large-scale high cell density production where the cost of compressed air becomes a significant factor [22].

High-speed centrifugation on a tubular separator at 18,000 × g quickly and efficiently clarified the culture from cells. The flow rate of 1.5 L/m turned out to be optimal; at this rate, the residual content of cells did not exceed 1 g/L. Measurements of activity before and after centrifugation of the culture showed that the changes did not exceed 10 units/mL, which indicates that these centrifugation modes did not damage the chymosin.

For better preservation of the culture, it must be sterilized. The chymosin is not thermostable protein and it have been sterilized by filtration through 0.22 μm. The large volume does not allow the use of dead-end filtration and the use of crossflow filtration is preferred. The Vladisart UPIRO-018 filtration unit developed for this purpose based on modular filters was effective. 17 liters of culture was completely filtered within 3.5 hours. Losses amounted to no more than 1% (Table). The next step was the activation of chymosin. The enzyme expressed in yeast is the inactive form of chymosin – prochymosin. The inactive proenzyme contains an N-terminal progresment of 42 residues which is removed upon secretion into the acidic environment of the stomach, thereby leading to activation [28]. Changing the pH from 5.5 to 3.0 increased the activity of the recombinant enzyme from 174 units/mL to 225 units/mL (**Table 1**).

Concentration of the culture by cross-flow ultrafiltration allowed the volume to be reduced before chromatographic purification. The calculated mass of chymosin without propeptide is 35.6 kDa. The molecular weight of glycosylated recombinant camel chymosin is 42 kDa. A membrane with a cutoff of 10 kDa was used to prevent protein loss at this stage. Using cross-flow ultrafiltration, the total volume was concentrated 5.6 times. Losses in activity at this stage amounted to no more than 4 %. The culture was chilled after being heated during the ultrafiltration process.

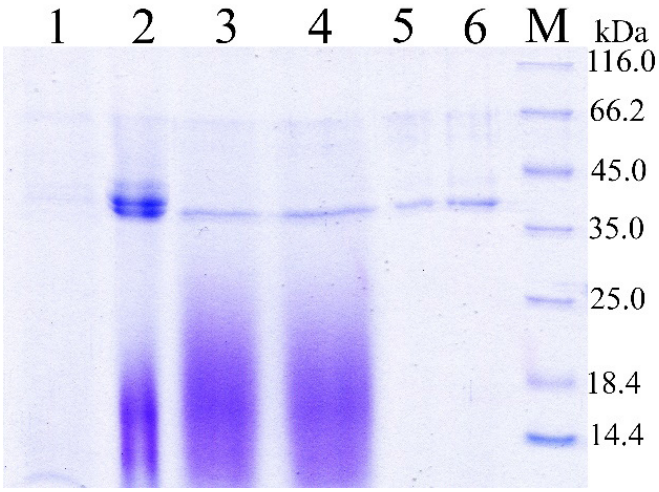

Figure 3. Chromatography purification of recombinant camel chymosin.

Lanes: 1 - Cultural media after 120h; 2 – Sample after ultrafiltration through 10 kDa membrane; 3,4 – SP-sepharose purified fractions; 5,6 – Q-sepharose purified fractions.

Chromatographic purification of recombinant camel chymosin included pre-filtration through 0,22 μm filter.

A more convenient way to store and transport enzyme preparations is in powder form. Chymosin is a thermolabile enzyme and loses its milk-clotting activity when heated above 50°C [10]. Therefore, an effective way to obtain a dry form of the enzyme is the sublimation [29]. Proteins need the correctly folded structure to maintain their properties [30], which can be broken during the freezing process. To stabilize the correct conformational state, the liquid solution of recombinant camel chymosin have to contain additional peptides. The casein peptone was used for this. The second reason for use casein peptone was to decrease enzymatic activity of camel chymosin per one gram. The high specific activity of purified camel chymosin causes difficulties in its use. The yield of sublimated recombinant camel chymosin was 38 grams with an activity of 36 000 U/g and moisture 5 %. The activity test showed that as a result of lyophilization, the total loss in activity was 56 %.

The results showed that both crude and purified supernatant after fermentation can be used to coagulate milk. [10,

Table 1. Information on activity at all stages of obtaining recombinant camel chymosin

Stage	Volume (L)	Milk-clotting activity	Total (%)	Losses (%)
Flask culture			-	-
Bioreactor culture (final)	17.5	174.5 U/mL	100 %	-
Culture after centrifugation	17	174 U/mL	96.9 %	3.1 %
Culture after microfiltration	17	174 U/mL	96.9 %	3.1 %
Culture after activation	17	225 U/mL	125 %	-
Culture after ultrafiltration	3	1240 U/mL	121,8 %	-
Sample after DEAE-Sepharose purification	3	990 U/mL	97.3 %	2.7 %
Sample after Q-Sepharose purification	0.291	4700 U/mL	44.8%	55.2 %
Powder (after lyophilization with peptone casein)	38 g	36 000 U/g	44.8%	55.2 %

11, 31, 32]. However, for use in commercial cheese production, chymosin must be purified and concentrated to reduce the amount of enzyme added [31]. And lyophilization allows you to increase the shelf life of the enzyme and simplify the calculation of its dosage [29].

The Pichia pastoris strain GS115/pGAPZA/ProchymCB was used to successfully produce recombinant camel chymosin in the current study. When the producer strain was cultured in a pilot bioreactor, it secreted recombinant prochymosin, which was converted to chymosin and isolated using a multistage purification process. A milk clotting enzyme suitable for use in the enzymatic coagulation of milk was produced by lyophilization with casein hydrolysate.

The proposed scheme for obtaining recombinant camel chymosin seems to be effective and suitable for producing a milk-clotting enzyme. The process can be scaled up and bring the enzyme production technology to the industrial level.

CONCLUSION

The *Pichia pastoris* GS115/pGAPZαA/ProchymCB strain was fermented in a pilot 50-liter fermenter by submerge fermentation. Using micro-, ultrafiltration in combination with ion-exchange chromatography, recombinant camel chymosin was purified. The yield of purified chymosin from 17 liters of culture of the recombinant strain was 1 368 000 units. By sublimation drying, a powder form of this enzyme was obtained, which showed high coagulation activity in cow and goat's milk. The proposed technology for obtaining recombinant camel chymosin can be used to obtain a milk-clotting enzyme.

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ПОЛУЧЕНИЕ РЕКОМБИНАНТНОГО ВЕРБЛЮЖЬЕГО ХИМОЗИНА МЕТОДОМ ГЛУБИННОЙ ФЕРМЕНТАЦИИ В ПИЛОТНОМ БИОРЕАКТОРЕ

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АННОТАЦИЯ

В сыроделии химозин используется в качестве молокосвертывающего фермента. Обладая высокой удельной активностью в отношении к-казеина, химозин превосходит другие протеолитические ферменты. Химозин двугорбого верблюда имеет более высокую молокосвертывающую активность, чем химозин теленка. Предложена схема получения молокосвертывающего препарата на основе рекомбинантного верблюжьего химозина. Глубинную ферментацию рекомбинантных дрожжей *Pichia pastoris* проводили в 50-литровом биореакторе и получали рекомбинантный верблюжий химозин. Активность химозина в культуре дрожжей составила 174,5 ед/мл. Химозин концентрировали в 5,6 раз с помощью ультрафильтрации с поперечным потоком с мембраной с отсечкой 10 кДа и очищали химозин с помощью ионообменной хроматографии. Активность очищенного химозина составила 4700 ед/мл. Сублимационной сушкой с казеиновым пептоном получен порошок химозина с активностью 36000 ед/г. Предложенная схема получения молокосвертывающего препарата на основе рекомбинантного верблюжьего химозина методом глубинной ферментации рекомбинантных дрожжей имеет перспективу использования на биотехнологических предприятиях.

Ключевые слова: химозин, ферментация, верблюд, теленок, биореактор, сублимационная сушка.

ПИЛОТТЫҚ БИОРЕАКТОРДА ТЕРЕҢ АШЫТУ АРҚЫЛЫ РЕКОМБИНАНТТЫ ТҮЙЕ ХИМОЗИНІН АЛУ

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ТҮЙІН

Ірімшік жасау өнеркәсібінде химозин сүтті ұйытатын фермент ретінде қолданылады. Басқа протеолитикалық ферменттерге қарағанда, химозиннің к-казеинге арнайылық белсендігі жоғары болып келеді. Бактриан түйесінің химозині сүтті ұюту белсенділігі бойынша бұзау химозиніне қарағанда жоғары. Осы жұмыста рекомбинантты түйе химозинінің негізінде сүтті ұйытатын препаратты алу схемасы ұсынылған. 50 литрлік биореакторда рекомбинантты *Pichia pastoris* ашытқысының тереңділік ашытуы жүргізіліп, рекомбинантты түйе химозині алынды. Ашытқы дақылындағы химозиннің белсенділігі 174,5 бірлік/мл құрады. Химозин 10 кДа бөлуші мембранасы бар айқас ағынды ультрафильтрация арқылы 5,6 есе концентрацияланып, кейін химозин ион алмасу хроматографиясы арқылы тазартылды. Тазартылған химозиннің белсенділігі 4700 бірлік/мл тең болды. Казеин пептонымен сублимациялық кептіру арқылы 36000 бірлік/г белсенділікпен ұнтақ күйінде химозин алынды. Рекомбинантты ашытқыларды тереңділік дақылдандыру арқылы рекомбинантты түйе химозині негізінде сүтті ұйытатын препаратты алудың ұсынылып отырған схемасы биотехнологиялық кәсіпорындарда қолдану мүмкіндігіне ие.

Негізгі сөздер: химозин, ашыту, түйе, бұзау, биореактор, сублимациялық кептіру