Production and Purification of Fungal Milk Clotting Enzyme from *Aspergillus candidus*

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Abstract: The search for rennet substitutes such as microbial rennet has increased fold due to increase in the demand for cheese products. Microbial rennet covers about one-third of the cheese consumption worldwide. Hence it is important to develop commercially viable and cost efficient method for purification of rennet from microbial sources. Hence the present work was focused on the production and purification of microbial rennet from *Aspergillus candidus*. The rennet was purified using a two step purification process involving solvent precipitation and chromatographic separation. The purity of the milk clotting enzyme was increased by 7.43 fold by solvent precipitation using equal mixture of 150% (v/v) ethanol-acetone. Then the enzyme was further purified using Diethylaminoethyl (DEAE) cellulose chromatography and 10.45 fold increase in enzyme activity was obtained after purification. The temperature of 35°C and substrate concentration of 0.25 mg/ml were found as optimum for maximum enzyme activity. The kinetics of the purified enzyme was studied and the Michaelis-Menten parameters such as rate constant (Km) and the maximum reaction rate (Vmax) were found as 0.059 mg/ml and 8.59×10^{-3} mmol/ml/sec respectively.

Keywords: Microbial rennet, Precipitation, Purification, Enzyme kinetics.

1. INTRODUCTION

Global consumption of rennet is predicted to be 21 million tonnes by the year 2015. An increase in worldwide demand for rennet coupled with reduced supply of calf rennet, has led to a search for microbial rennet [1,2]. Microbial rennet has several advantages over animal rennet. There are many sources of enzymes, ranging from plants, fungi, and microbial sources, that will substitute animal rennet. They are easy to generate and purify and do not rely on the availability of animal material. One-third of the rennet used at present for cheese making worldwide is from microbial source [3,4]. *Aspergillus niger* isolate was reported for the production of extracellular milk clotting enzyme. It was reported that ammonium sulphate was unsuitable for precipitation while the other precipitants produced sufficient active fractions [5].

Penicillium citrinum 805 was reported to produce rennin like milk clotting enzyme. The enzyme was partially purified using ammonium sulphate, ethanol and acetone as precipitating agents. The ammonium sulphate and ethanol were reported unsuitable for precipitation of enzyme into active fractions. The acetone was reported to produce active enzyme fractions [6]. The milk clotting enzyme was produced by solid state fermentation of Mucor varians pispek using wheat bran as substrate and it was reported that 4000 MKU/g of wheat bran was produced. The crude culture extract was precipitated using ammonium sulphate, mixing with ethanol, methanol or acetone [7]. Seven fold purification and 22% of final recovery of microbial rennet was obtained from culture filtrate of Rhizomucor miehei using two step purification process using ion-exchange and affinity chromatography. The purified enzyme was free of brown pigmentation and it was stable and active at acidic pH and its thermostability was comparable to calf rennet [8].

Myxococcus xanthus 422 was reported to produce extracellular milk-clotting enzyme. The enzyme was purified by anion-exchange chromatography into two active fractions. The molecular mass of the enzyme was reported as 58.2 kDa using SDS-PAGE. The purified protein fraction II was reported to exhibit significantly higher milk clotting activity [9]. The fruiting bodies Pleurotus ostreatus was reported to produce milk clotting enzyme. The enzyme was precipitated by ammonium sulphate and purified using gel chromatography on Sephadex G75, and ion exchange chromatography on carboxy methyl cellulose [10].

The thermophilic fungus Thermomucor indicae-*seudaticae* was reported to produce milk clotting enzyme [11]. Purification of the milk clotting enzyme from *Bacillus amyloliquefaciens* D4 was reported to increase the purity by 17.2 fold with 20% recovery by ammonium sulphate precipitation followed by ion-exchange chromatography separation [12]. Thus the purification of milk clotting enzyme from microbial source is a complex process involving various unit operations and this increase cost of the purified enzyme. Therefore it is important to develop a low cost purification steps to purify the milk clotting enzyme.

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Thus the present work was focused on the production and purification of milk clotting enzyme from *Aspergillus candidus.*

2. MATERIALS AND METHODS

2.1. Microorganism

Aspergillus candidus MTCC 1989 was obtained from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India.

2.2. Substrate Preparation

The soybean meal was dried at 98˚C and finely powdered. The powdered material was sieved using 80/120 mesh. The sieved soybean meal flour (SBMF) retained in 120 mesh was used as substrate for milk clotting enzyme production.

2.3. Preparation of Stock and Inoculum Culture

Stock culture of *A. candidus* MTCC 1989 strain was cultivated in yeast extract agar slants at 25˚C for 7 days, stored at 4˚C and sub cultured periodically. Then it was sub cultured in skimmed milk powder-yeast extract agar slants at 25˚C for 5 days and used as innoculum for milk clotting enzyme production.

2.4. Production of Milk Clotting Enzyme

The production media of 150 ml was prepared in 500 ml flasks. The composition (w/v) of the media was yeast extract 1.5%, skimmed milk powder 0.87%, SBMF 1%, glucose 0.5%, potassium dihydrogen phosphate 0.11%, magnesium sulphate 0.05% and Mineral salt solution 150 µl. The initial pH was adjusted to 6.1 and autoclaved. The autoclaved fermentation media was inoculated with 2% innoculum, incubated in a temperature controlled shaker at 30°C and 150 rpm for 5 days. The entire fermentation broth was filtered using Whatman No. 1 filter paper to obtain the crude enzyme and refrigerated at 4˚C for further purification and characterization.

2.5. Precipitation of Milk Clotting Enzyme

The crude enzyme was precipitated using ammonium sulphate, ice cold acetone, ice cold ethanol and mixture of ice cold acetone-ethanol. 1 ml of the crude enzyme was mixed with ammonium sulphate. The mixture was mixed for 30 min and kept at 4°C for overnight. The precipitate was collected by centrifugation at 4°C and 10,000 rpm. Then the precipitate was suspended in 1 ml of 0.01 M phosphate buffer (pH-8.5). The milk clotting activity and protein concentration were estimated. Then the same procedure was repeated independently using 1.5 ml of ice cold acetone, 1.5 ml of ice cold ethanol and equal mixture 1.5 ml of ice cold acetone and ice cold ethanol.

2.6. Purification of Milk Clotting Enzyme

The partially purified milk clotting enzyme obtained by precipitation was further purified using ion-exchange chromatography on DEAE-cellulose column. The column was equilibrated with 50 mM phosphate buffer (pH 8.5). The partially purified milk clotting enzyme was loaded to the column and the column was washed using wash buffer. Then the column was eluted using elution buffer with varied concentration of sodium chloride (0.05 to 0.5 M). The eluted protein fractions were collected and analyzed for its milk clotting activity and protein concentration [13].

2.7. Effect of Temperature and Substrate Concentration on Milk Clotting Activity

The effect of temperature on milk clotting activity of the purified enzyme was studied at various temperatures from 25 to 50°C with an internal of 5°C under standard conditions. The standard skimmed milk powder was used as substrate for milk clotting enzyme activity. The effect of substrate concentration was studied by varying the concentration from 0.05 to 0.3 mg/ml with an interval of 0.05 mg/ml.

2.8. Estimation of Milk Clotting Activity

Calcium chloride-skimmed milk powder solution was prepared by dissolving 0.11g of calcium chloride and 5g of skimmed milk powder in 100 ml of distilled water. The Calcium chloride-skimmed milk powder solution was pre-incubated for 10 min at 35˚C. 5 ml of $CaCl_2$-SMP solution was added to 0.5 ml of enzyme and the time of clotting of milk was noted. The Milk Clotting Activity was determined using the relation, MCA = (2400*5D) / (0.5T), where, “D” is the dilution factor and “T” is the milk clotting time in second. The unit of milk clotting activity was defined in terms of Milk Clotting Unit (MKU). One MKU is defined as the amount of enzyme required to clot 1 ml of a solution containing 0.1 g skim milk powder and 0.00111 g calcium chloride in 40 min at 35°C [14].

2.9. Estimation of Protein by Lowry’s Method

Lowry’s method for protein estimation is the widely accepted method for accurate determination of protein

concentration. GENEI's protein estimation kit, cat # KT–18 is based on this method. It is a ready to use reagent kit for rapid estimation of protein with ease and consistency [15].

3. RESULTS AND DISCUSSION

3.1. Partial Purification of Milk Clotting Enzyme by Precipitation

The produced milk clotting enzyme was precipitated using various precipitating agents such as ammonium sulphate, ethanol, acetone and the mixture of ethanol and acetone. The 150 %(v/v) of ice cold ethanol, 150 %(v/v) of ice cold acetone, a mixture of 75 %(v/v) ice cold ethanol and 75 %(v/v) of ice cold acetone, and 3.9 g of ammonium sulphate were added to 100 ml the crude milk clotting enzyme produced by *A. candidus*. Then the protein was precipitated, pelleted and re-suspended in phosphate buffer. The concentrations of protein in each of the four samples were analyzed using Lowry's method. The milk clotting activity was calculated for each of the four fractions of partially purified protein and then the specific milk clotting activity was calculated. The specific milk clotting activity of the different precipitates were compared with the specific activity of crude enzyme.

It was observed that a protein concentration of 0.008 mg/ml was obtained by ethanol-acetone precipitation with milk clotting activity of 117.07 MCU/ml. The specific milk clotting activity of 7549, 14466, 15190, 8878 MCU/mg were obtained from ethanol, acetone, ethanol and acetone mixture and ammonium sulphate precipitates respectively (Table **1**). It was found that the protein purified by ethanol-acetone mixture had the highest specific activity of 15190 MCU/mg of protein (Figure **1**). Hence it was observed that the 150% (v/v) of ethanol-acetone mixture precipitated the crude enzyme with the maximum purity. The crude milk clotting enzyme was purified by 7.45 fold using ethanol-acetone mixture as precipitating agent. It was reported in literature that the ammonium sulphate was unsuitable for precipitation of extracellular milk clotting enzyme *from Aspergillus niger* isolate [5]. The ammonium sulphate and ethanol were reported unsuitable for precipitation of milk clotting enzyme from *Penicillium citrinum* into active fractions and the acetone was reported to produce active enzyme fractions [6]. Thus the equal mixture of ethanol-acetone can be effectively used for milk clotting enzyme precipitation.

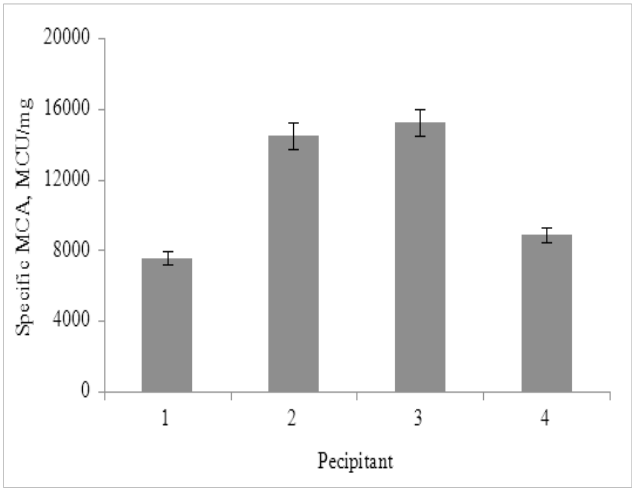

Figure 1: Specific milk clotting activity of enzyme precipitates (1-Ice cold Ethanol; 2-Ice cold Acetone; 3-Equal volume of ice cold Ethanol and ice cold Acetone; 4-Ammonium sulphate).

3.2. Purification of Milk Clotting Enzyme using Ion Exchange Chromatography

The partially purified enzyme after ethanol-acetone precipitation was further purified using ion exchange chromatography using DEAE-cellulose column. All the eluted fractions were analyzed for protein concentration and milk clotting enzyme activity. The specific milk clotting activity of all the fractions were calculated and

Table 1: Protein Concentration and Milk Clotting Activity of Precipitated Enzyme

Sl. No.	Precipitant	Protein Concentration, mg/ml	Milk Clotting Activity, MCU/ml	Specific Milk Clotting Activity, MCU/mg	Fold purification
1	Ethanol	0.058	436.36	7549.09	3.70
2	Acetone	0.013	195.12	14466.89	7.10
3	Ethanol + Acetone	0.008	117.07	15190.24	7.45
4	Ammonium Sulphate	0.029	260.87	8878.12	4.35
5	Crude enzyme	0.369	750.00	2037.96	-

compared. It was observed from the purification results that the maximum specific milk clotting activity of 21292 MCU/mg was obtained in fraction number 5 (Figure **2**). The enzyme was purified by 10.45 fold after purification using ion exchange chromatography when compared to the milk clotting activity of the crude enzyme. The purity of milk clotting enzyme obtained is comparable with seven fold purification of microbial rennet from *Rhizomucor miehei* reported in literature using two step purification process using ion-exchange and affinity chromatography [8].

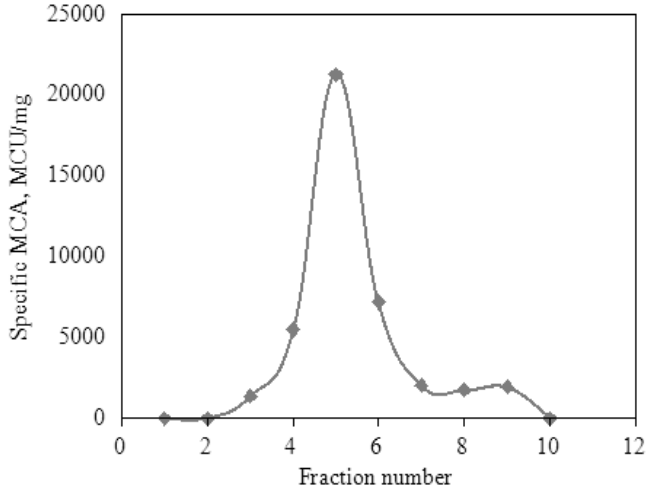

Figure 2: Specific milk clotting activity of various enzyme fractions after ion exchange chromatographic separation.

3.3. Effect of Temperature on Milk Clotting Activity

The effect of temperature on milk clotting activity of purified enzyme was studied and reported in Figure **3**. It was observed that the milk clotting activity of purified enzyme was increased with increase in temperature upto 35°C. It was observed that the milk clotting activity was decreased with increase in temperature from 35°C to higher temperature. The maximum milk clotting activity of 296.29 MCU/ml was observed at 35 to 50°C. Hence the optimum temperature for maximum milk clotting activity was found as 35°C. Thus the enzymes are found sensitive to temperature, as temperature increases the activity is also expected to increase up to a particular temperature and enzyme denaturation occurs at higher temperature.

3.4. Effect of Substrate Concentration and Kinetics of Milk Clotting Enzyme Activity

The Michaelis-Menten kinetics for steady state enzyme catalyzed reaction is given by eq. (1).

$$v = v_{\max}([S]/K_m + [S]) \quad (1)$$

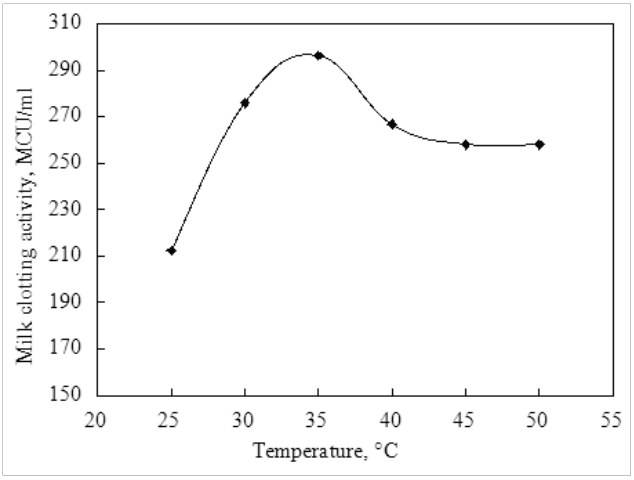

Figure 3: The effect of temperature on purified milk clotting enzyme activity.

where V is the reaction velocity (the reaction rate), K_m is the Michaelis–Menten constant, $V_{\max}$ is the maximum rate of the reaction, and [S] is the substrate concentration. The Lineweaver–Burk plot is a graphical representation of the Lineweaver–Burk equation (eq**.** 2) of enzyme kinetics, described by Hans Lineweaver and Dean Burk in 1934 [16]. The Lineweaver–Burk plot is a useful graphical method of determination of Michaelis–Menten parameters.

$$1/v = \{K_m/v_{\max} * 1/[S]\} + 1/v_{\max} \quad (2)$$

The *y*-intercept of such a graph is equivalent to the inverse of $V_{\max}$; the *x*-intercept of the graph represents $-1/K_m$. Lineweaver–Burk plot also gives a quick, visual impression of the different forms of enzyme inhibition. Lineweaver–Burk plot provides a more precise way to determine V_{max} and K_m. V_{max} is determined by the point where the line crosses the $1/V_i$ = 0 axis (so the [S] is infinite). K_m equals V_{max} times the slope. This is easily determined from the intercept on the X axis [17,18]. The effect of initial substrate concentration on milk clotting activity of purified enzyme was studied and fitted with the Michaelis-Menten kinetic model as shown in Figure **4**. The maximum rate of milk clotting activity of 7.5 $\times 10^{-3}$ mmol/ml/sec was observed at 0.25 mg/ml of substrate concentration. The Lineweaver–Burk plot was constructed using reciprocal of the substrate concentratin (X-axis) and reciprocal of the rate of enzyme action. The Michaelis–Menten kinetic parameters such as maximum rate milk clotting action (V_{max}) and affinity of substrate (K_m) were determined from Lineweaver-Burk plot shown in Figure **5**. The Michaelis–Menten constant of the purified milk clotting enzyme was (Km) 0.059 mg/ml and the maximum reaction rate of milk clotting enzyme was (V_{max}) 8.59×10^{-3} mmol/ml/sec.

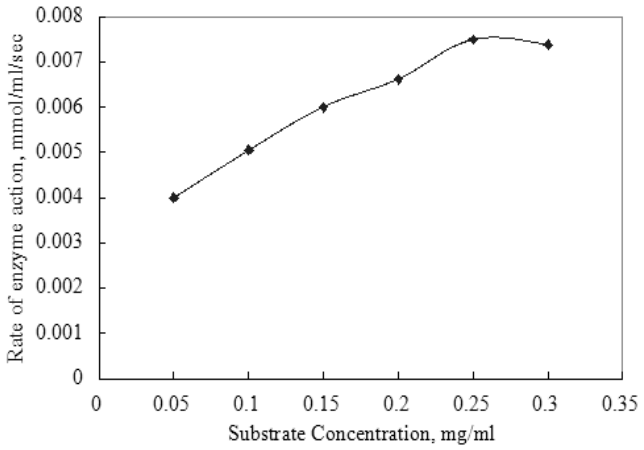

Figure 4: Rate of milk clotting enzyme action at different substrate concentration.

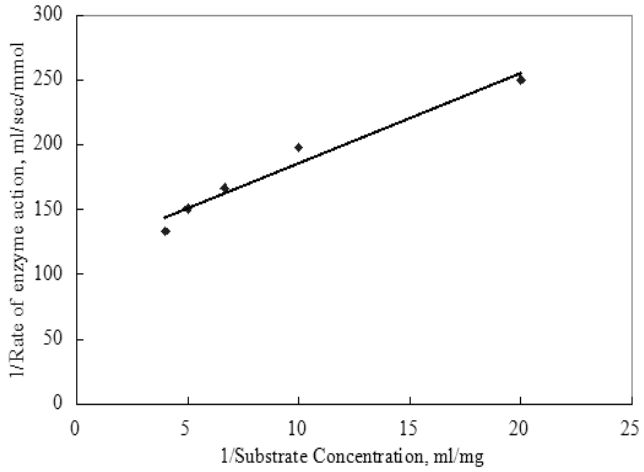

Figure 5: Lineweaver-Burk plot for the determination Michaelis–Menten Parameters.

4. CONCLUSIONS

The fungal milk clotting enzyme was produced from Aspergillus candidus and was purified using mixed solvent precipitation and ion exchange chromatography. The crude enzyme was partially purified in mixed solvent precipitation to 7.45 fold, which has reduced the sample load to the ion exchange chromatography for final purification. Finally 10.45 fold increase purity was obtained using ion exchange chromatography with DEAE Cellulose column. The purified enzyme has optimal temperature of 35°C for maximum milk clotting activity. The Michaelis–Menten was parameters were found as (Km) 0.059 mg/ml and the maximum reaction rate of milk clotting enzyme was (V_{max}) 8.59×10^{-3} mmol/ml/sec. The lower Michaelis–Menten constant of the purified milk clotting shows that the enzyme has higher affinity towards the substrate.

REFERENCES

[1] Ruchi R. Cheese-Global Consumption. Worldwide Cheese Consumption Patterns. 2010.

[2] Bernardinelli SE, Bottaro Castilla HR, Waehner, RS, Muse J, Fraile ER. Production and properties of the milk-clotting enzyme. Rev Argent Microbiol 1983; 15: 95-104.

[3] Fox PF, Paul M, Timothy MC and Timothy PG. Diversity of cheese varieties: An overview. Cheese: Major cheese groups 2004; 1-23.

[4] Poza M, Sieiro C, Carreira L, Barros VJ, Villa TG. Production and characterization of the milk-clotting protease of *Myxococcus xanthus* strain 422. J Ind Microbiol Biotechnol 2003; 30: 691-98. http://dx.doi.org/10.1007/s10295-003-0100-y

[5] Osman HG, Abdel-Fattah AF, Abdel-Samie M and Souhair SM. Production of a Milk-clotting Enzyme Preparation by *Aspergillus niger* and the Effect of Various Factors on its Activity. J Gen Microbiol 1969; 59:125-29. http://dx.doi.org/10.1099/00221287-59-1-125

[6] Abdel-Fattah F, Souhair SM and El-Hawwary NM. Production and Some Properties of Rennin-like Milk-clotting Enzyme from *Penicillium citrinum.* J Gen Microbiol 1971; 70:151-55. http://dx.doi.org/10.1099/00221287-70-1-151

[7] Bernardinelli SE, Bottaro Castilla HR, Waehner, RS, Muse J and Fraile ER. Production and properties of the milk-clotting enzyme, Rev Argent Microbiol 1983; 15 (2): 95-104.

[8] Preetha S, Boopathy R. Purification and characterization of a milk clotting protease from *Rhizomucor miehei.* World J Microb Biotechnol 1997; 13: 573-578. http://dx.doi.org/10.1023/A:1018525711573

[9] Poza M, Sieiro C, Carreira L, Barros VJ and Villa TG. Production and characterization of the milk-clotting protease of *Myxococcus xanthus* strain 422. J Ind Microbiol Biotechnol 2003, 30: 691–98. http://dx.doi.org/10.1007/s10295-003-0100-y

[10] Lebedeva GV and Proskuryakov MT. Purification and Characterization of Milk-Clotting Enzymes from Oyster Mushroom (*Pleurotus ostreatus* (Fr.) Kumm.). App Biochem Microbiol 2009; 45 (6): 623–25. http://dx.doi.org/10.1134/S0003683809060088

[11] Carolina MD, Eleni G, Mauricio B and Roberto S. Production and characterization of a milk-clotting protease in the crude enzymatic extract from the newly isolated *Thermomucor indicae-seudaticae* N31. Food Chem 2010; 120: 87–3. http://dx.doi.org/10.1016/j.foodchem.2009.09.075

[12] Xiaoling H, Fazheng R, Huiyuan G, Weibing Z, Xi S and Bozhong. Purification and properties of a milk-clotting enzyme produced by *Bacillus amyloliquefaciens* D4. Korean J Chem Eng 2011; 28(1): 203-208. http://dx.doi.org/10.1007/s11814-010-0347-8

[13] Rashbehari T, Binita S, Rintu B. Purification and characterization of a protease from solid state cultures of *Aspergillus parasiticus.* Process Biochem 2003; 38: 1553-58. http://dx.doi.org/10.1016/S0032-9592(03)00048-7

[14] Arima K, Iwasaki S, Tamura G. Milk clotting enzyme from microorganisms. 1. Screening tests and identification of the potent fungus. Agric Boil Chem 1967; 31: 540-54. http://dx.doi.org/10.1271/bbb1961.31.540

[15] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265-75.

[16] Lineweaver, H and Burk, D. The Determination of Enzyme Dissociation Constants. J Am Chem Soc 1934; 56 (3): 658–66. http://dx.doi.org/10.1021/ja01318a036

[17] Hayakawa K, Guo L, Terentyeva EA, Li XK; Kimura H, Hirano M, Yoshikawa K, Nagamine T, Katsumata N, Ogata T,

Tanaka T. Determination of specific activities and kinetic constants of biotinidase and lipoamidase in LEW rat and Lactobacillus casei (Shirota). J Chromatogr B Analyt Technol Biomed Life Sci 2006; 844(2): 240–50. http://dx.doi.org/10.1016/j.jchromb.2006.07.006

[18] Baskar G, Arulluca Sundarsingh S, Bharath Kumar S, Muthukumaran C, Renganathan S. Optimization and Kinetics of Enzymatic Hydrolysis of Cassava Starch by Cross-Linked Fungal α–Amylase. Asian J Microbiol Biotechnol Environ Sci 2009; 11(2): 323-26.

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