Vol. 6 No. 1
Winter 1995

PARTIAL PURIFICATION AND CHARACTERIZATION OF ß-GALACTOSIDASE FROM *ASPERGILLUS NIGER* UV-5

I. Rasouli and P. R. Kulkarni*

Food and Fermentation Technology Division, University of Bombay, Department of Chemical Technology, Matunga Road, Bombay-400 019, India

Abstract

The enzyme β-galactosidase from a mutant strain of *A. niger* UV-5 was partially purified using ammonium sulfate and acetone. The saturation range of 60-80% ammonium sulfate was found to yield 60.5% enzyme recovery with 2.4 fold purification. Acetone precipitation at enzyme: acetone ratio of 1 : 1.5 brought about a higher yield i.e. 68% and three-fold purification. The combined procedures of 1.5 volume solvent fractionation followed by 50% ammonium sulfate precipitation brought about 8-fold purification with 40% enzyme yield. The optimum temperature of the enzyme was 65°C and the optimum pH was 4-5. The β-galactosidase was strongly inhibited by galactose. Comparative study of partially purified β-galactosidase in the present study with a commercial lactase from *A. oryzae* revealed comparable results.

Introduction

Purification of enzymes can be carried out to various degrees. The degree of purity of the product is usually related to its intended use. Very high purification of enzymes is usually recommended only when the enzyme has special uses like therapeutic and medicinal applications [1]. In most other applications, partial purification is sufficient. It is well known that as the purification increases, the yield decreases which results in an increase in the cost of the enzyme.

β-galactosidases from different sources are known to exhibit a range of properties. The enzyme from *K. fragilis* and several bacterial cultures have been reported to be heat labile [2, 3]. Molds have been reported to produce relatively heatstable β-galactosidase [4].

Considering the variations in the properties of the enzyme, and with a view to studying the extent of applicability of β-galactosidase, it was planned to partially purify and study the properties of β-galactosidase produced by the mutant strain of *A. niger* NCIM-616 (i.e. *A. niger* UV-5).

Keywords: β-galactosidase; Lactase; Partial purification; *A. niger;* Mutant

Materials and Methods

Materials

Folin Ciocalteus reagent and *o*-nitro phenyl-β-D-galactopyranoside, ONPG (SRL), lactose, glucose and galactose (Hi-media) and all other chemicals (Loba, analytical reagent grade) were used. Commercial lactase (*A. oryzae*) was obtained from M/S Rathi Papain Ltd., Bombay. Cheese whey was from Aarey dairy, and whole milk was purchased locally.

Methods

Preparation and Assay of β-Galactosidase

The mutant strain of *A. niger* UV-5 obtained by chemical treatment followed by UV irradiation in our laboratory was grown in shake flasks containing a primarily optimized medium composed of (wt/v), 3% wheat bran, 0.2% $(NH_4)_2SO_4$, 0.6% $NaNO_3$, 0.1% lactose, 1% KH_2PO_4, 1% KCl, and 0.5% $(NH_4)_2HPO_4$ at 30°C for 72 hours. The clear broth filtrate was used as crude β-galactosidase and assayed using the method of Nevalainen [5]. Protein was assayed by the method of Lowry *et al.* [6].

Partial Purification of β-Galactosidase with Ammonium Sulfate

Partial purification of β-galactosidase from the crude culture filtrate was done by adding appropriate quantities [7] of ammonium sulfate at 4°C to obtain different fractions (i.e. 0-40, 40-60 and 60-80%).

Precipitation Using Acetone

To 100 ml of crude culture filtrate held at 0°C was added chilled extra pure acetone with constant stirring. Filtrate to solvent ratios used were 1:1, 1:1.5. The precipitate formed was centrifuged at 13,000 × g for 15 minutes. The precipitate was dried by final washing with ether and stored in a desiccator. It was then dissolved in 10 ml of 0.075 M sodium acetate buffer pH-4, and assayed for β-galactosidase activity and protein content.

Purification Using Combinations of Acetone and Ammonium Sulfate Precipitation Procedures

The enzyme was first precipitated by acetone at 1:1.5 broth:solvent ratio. The precipitate obtained from 400 ml extract was dissolved in 100 ml sodium acetate buffer and subjected to ammonium sulfate precipitation at different saturation levels.

Deproteinization of Cheese Whey and Estimation of Lactose Content of Milk and Whey

Deproteinization was carried out by heating whey at 90°C for 15 minutes followed by filtration. Lactose was estimated as described by Nickerson *et al.* [8].

Determination of Optimum pH and Temperature of β-Galactosidase from *A. niger* UV-5

The enzyme assay was carried out at different temperatures using 1.7 mM ONPG prepared in buffers of various pH values ranging from 3-8 viz: 100 mM McIlvaine pH 2.4-7.7; 100 mM Kolthoff pH 8-11. The pH and temperature at which maximum hydrolysis occurred was taken as the optimum pH and temperature of β-galactosidase.

Determination of pH and Thermal Stability of β-Galactosidase from *A. niger* UV-5

2 ml of β-galactosidase (specific activity 1453) was incubated in 2 ml buffers of pH 3 to 8 as above for 60 minutes at 30°C. Using ONPG, the residual activities with respect to control (i.e. 100%) were calculated. The buffers used were: 100 mM McIlvaine buffer pH 2.4-7.7 and 100 mM Kolthoff buffer pH 8-11. The control consisted of 2 ml of soluble or 100 mg of immobilized enzyme incubated with 2 ml of 0.075 M sodium acetate buffer pH-4.5 (i.e. the optimum pH of the enzyme) for 60 minutes at 30°C.

5 ml of the β-galactosidases was held in a water bath at various temperatures ranging from 45°C-70°C for various time intervals between 10-30 minutes. The residual activities were measured using ONPG as the substrate at 45°C for 10 minutes and compared with the original activity.

Determination of the Effect of Metal Ions on the Activity of β-Galactosidase from *A. niger* UV-5

The assay system consisting of 0.2 ml of enzyme and 0.8 ml of 1.7 mM ONPG containing the effective concentrations of metal ion varying between 1×10^{-4} to 1×10^{-3} was incubated for 10 minutes at 45°C. The activity was estimated and compared with respect to a control tube without metal ions.

Determination of the Effect of Glucose and Galactose on the Activity of β-Galactosidase from *A. niger* UV-5

0.2 ml of a soluble enzyme solution with specific activity of 1453 was added to 0.8 ml of 1.7 mM ONPG containing various molarities of sugars and assayed for β-galactosidase activity as described in method 2, and compared with the assay system without sugar.

Comparative Study of β-Galactosidase Activity from *A. niger* UV-5 and a Commercial Lactase from *A. oryzae*

0.2 ml enzyme solution (1 mg/ml) was incubated with 5 ml of the substrates viz; 5% lactose pH-4.5, deproteinized cheese whey, and whole milk at 60°C for 6 hours.

Results and Discussion

In the present work, initially, an attempt was made to partially purify the β-galactosidase present in the culture filtrate obtained from fermentative action by *Aspergillus niger* (UV-5). Table 1 shows the β-

galactosidase activities recovered at different ammonium sulfate saturation fractions. It was observed that most of the activity was recovered in 60-80% ammonium sulfate fraction. At 60-80% saturation fraction, the specific activity was 440. The purification was 2.4-fold and more than 60% of the total β-galactosidase activity was recovered (Table 1).

β-galactosidase from different organisms has been precipitated by ammonium sulfate [9-11]. The extent of purity and activity yield differ from one organism to the other. Bhal and Agrawal [9] precipitated β-galactosidase from a strain of *A. niger* by 50% ammonium sulfate saturation to get 1.4-fold purification and 85% enzyme yield. Greenberg and Mahoney [11] subjected β-galactosidase from *S. thermophilus* to two successive 65% ammonium sulfate precipitation which resulted in final 2.4-fold purification and 81.7% yield. The purification of 2.4-fold (Table 1) obtained in the present study is similar to that of *S. thermophilus* reported above.

The protein and enzyme contents of β-galactosidase, followed by solvent precipitation using acetone, are shown in Table 2. It was observed that a solvent ratio of 1:1.5 showed 68% recovery of enzyme with a specific activity of 547 units/mg protein. Table 2 also shows that partial purification of β-galactosidase by precipitation with acetone (1:1.5) yielded a product with three-fold purification.

Acetone precipitation technique has been employed for β-galactosidase purification from different microbial sources [12-16]. Mahoney *et al.* [12] partially purified β-galactosidase from a strain of *K. fragilis* by acetone fractionation at the ratio of 1:1. More than 95% of enzyme activity with a 2.5-fold purification was achieved. Acetone precipitation at a ratio of 1:1 applied to a β-galactosidase preparation from *K. fragilis* yielded 97% of activity with 2.74-fold purification [13]. In the present study, the acetone precipitation at the enzyme: acetone ratio of 1:1.5 brought about 3-fold purification recovering 68% of total enzyme activity. At the increased solvent concentrations of 1:2 or 1:2.5 more than 95% of enzyme activity was recovered, however there was a fall in purification rate.

The purification of β-galactosidase by combined procedures of precipitation using 1:1.5 ratios of the enzyme broth to acetone followed by ammonium sulfate precipitation at different saturation levels is shown in Table 3.

As 1:1.5 (broth:acetone) ratio yielded the maximum recovery of 68% with better specific activity, the enzyme was first precipitated by acetone and then it was precipitated by different saturation levels of ammonium sulfate.

Of the different levels of ammonium sulfate i.e. 0-50%, 0-70%, and 0-90%, the highest purity was achieved by 50% ammonium sulfate. The effectiveness of purification steps is given in Figure 1. Ramana Rao and Dutta [4] could obtain 4-fold purification by acetone precipitation of *S.*

Table 1. Partial purification of β-galactosidase by ammonium sulfate precipitation

	Crude enzyme	Ammonium sulfate fractionation 0-40%	40-60%	60-80%
Volume (ml)	100	15	15	15
Total protein (mg)	160	50	60	40
Total activity (ONPG units)	29120	2280	8880	17600
Specific activity Units / mg protein	182	45	148	440
Purification fold	1	0.25	0.8	2.4
Yield (%)	100	7.8	30.5	60.5

Table 2. Partial purification of β-galactosidase by acetone precipitation

	β-galactosidase : Acetone fractionation				
	Crude	1 : 1	1 : 1.5	1 : 2	1 : 2.5
Volume (ml)	100	15	26	50	75
Total protein (mg)	160	25	36.2	57.6	74
Total activity (ONPG units)	29120	11648	19801	26208	28246
Specific activity Units / mg protein	182	466	547	455	382
Purification fold	1	2.6	3	2.5	2.1
Yield (%)	100	40	68	90	97

Table 3. Partial purification of β-galactosidase by combined procedure of acetone and ammonium sulfate precipitation

		Acetone	% Ammonium sulfate		
β-galactosidase ⇒	Crude	1 : 1.5	0-50	0-70	0-90
Volume (ml)	400	100	15	18	20
Total protein (mg)	640	145	32	58	85
Total activity (ONPG units)	116480	79206	46496	57188	63920
Specific activity Units / mg protein	182	547	1453	986	752
Purification fold	1	3	8	5.4	4.1
Yield (%)	100	68	40	49	55

thermophilus β-galactosidase. They were also able to increase the purity of acetone precipitated enzyme from 4-to 8-fold by 50% ammonium sulfate saturation that yielded 55% of the total activity. The results from this study are in agreement with the above report.

Using 1.7 mM ONPG the optimum temperature of partially purified β-galactosidase was determined and the results are summarized in Figure 2. The enzymes recorded the highest activity percentage at 65°C. The results are similar to those of *A. tenuis* [17]. The high optimum temperature discourages growth of

contaminants, this being an advantage of the enzyme under study.

Figure 3 shows the effect of pH on activity of β-galactosidase with ONPG. The optimum pH for the β-galactosidase was found to be around 5. In reaction with 1.7 mM ONPG at pH-8.0 the enzyme showed 50% loss of activity with respect to the optimum. The optimum pH of β-galactosidase in the present study falls in the range of the pH reported for fungal enzymes [18,19]. It has been suggested that such enzymes may be of value for the hydrolysis of lactose in high-acid products such as yoghurt or acid whey, or in concentrated products [18, 20].

The enzyme preparations were incubated at different temperatures for different time intervals and assayed for residual activities. The results are shown in Figure 4. The enzyme showed 100% activity for up to 20 minutes at 65°C. As expected, as the incubation time increased the enzyme activity decreased. The enzyme showed half the activity on exposure to 70°C for 20 minutes or 65°C for 25-30 minutes. β-galactosidase in the present study exhibited a good thermostability (Fig. 4) and can be contrasted with the stability of bacterial [4], yeast [21] and even some fungal [22-25] enzymes. The occurrence of proportional decrease in activity with increase in temperature (Fig. 4) suggests that there may be sequential denaturation of the structural components of the enzyme.

The effect of preincubation of β-galactosidase in media of various pH values on the enzyme stability is needed to get an idea of its application to substrates of various pH levels. The stability of different pH levels in 30 minutes reaction time at 30°C is shown in Figure 5. It was observed that when incubated with buffers of pH levels ranging between 3.2 to 5.4 for 30 minutes the enzyme still retained as much as about 90% of its activity. The stability range of pH suggests the use of β-galactosidase from *A. niger* UV-5 in acidic applications such as cheese whey.

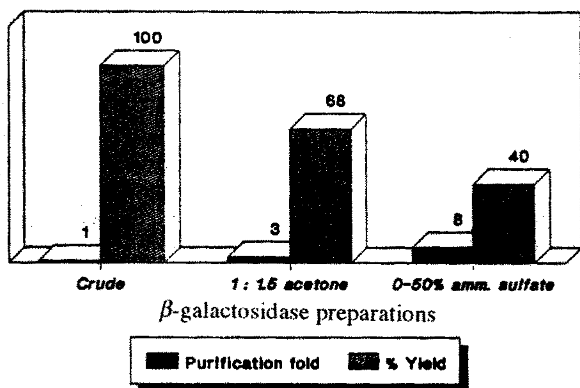


Figure 1. Effectiveness of purification steps

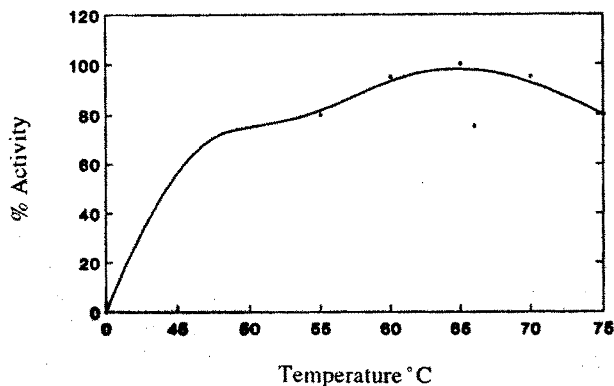


Figure 2. Effect of temperature on β-galactosidase activity

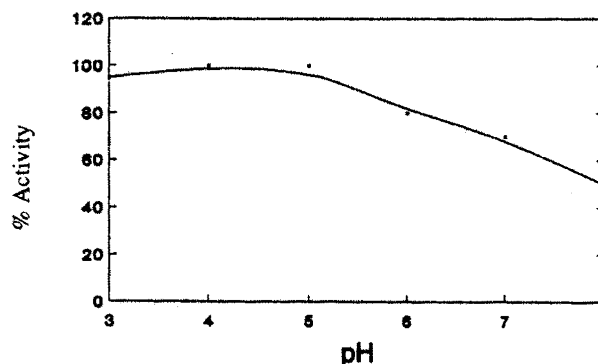


Figure 3. Effect of pH on activity of β-galactosidase from *A. niger* UV-5

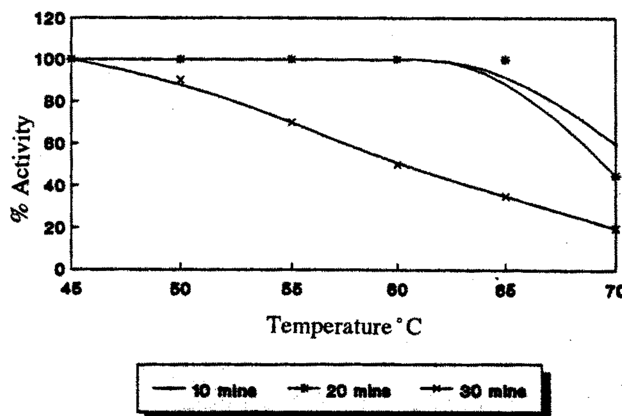
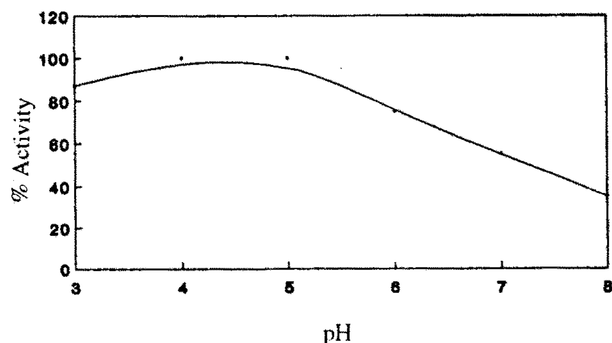


Figure 4. Temperature stability of β-galactosidase



The enzyme was incubated in buffers of different pH/60 minutes at 30°C.

Pre-incubation activity with 1.7 mM ONPG at pH 4.5 and temperature 45°C for 10 minutes is taken as 100%.

Figure 5. pH stability of β-galactosidase from *A. niger* UV-5

Table 4 shows the effect of different metal ions on soluble β-galactosidase activity. No significant activation or inhibition was observed in the presence of metal ions used at two different concentrations of 1×10^{-3} and 1×10^{-4} M. The metal ions Mg^{++} and Fe^{+++} are reported to activate bacterial β-galactosidase [26] while fungal lactase was reported to have no metal ion requirement for activity [20]. In the present work, addition of EDTA did not show any effect suggesting probable independence of the activity with respect to metal ions. At 1×10^{-4} M, $FeSO_4$, KCl, and $MnCl_2$ showed very slight stimulation but $MgSO_4$ did not have any effect. Mg^{++} was reported to inhibit β-galactosidase activity produced by *A. niger* [27]. The present study fails to agree with that report.

Galactose and glucose are known to influence the activity of β-galactosidase from some microbial sources such as *B. subtilis* [28], *L. Kefiranofaciens* [29], *C. laurentii* [21], *A. foetidus* [20], and *A. Oryzae* [13]. In the present study, in the case of soluble enzyme, the presence of 20 mM glucose had no effect but at as high as 200 mM, 78.8% activity was exhibited. In the case of galactose, however, at 20 mM only 22% activity was shown and at 200 mM 90% inhibition was observed. In the case of a combination of glucose and galactose at an effective total concentration of 20 mM, 67% inhibition and at 200 mM more than 85% inhibition was observed (Table 5). Galactose seems to be one of the end products inhibiting the enzyme activity. Wendroff and Amundson [30] noted 7% and 60% inhibition of β-galactosidase from *K. fragilis* by galactose and glucose, respectively. The results from the present study do not favour the above report, probably because of the difference in the enzyme source. In the present study, galactose was found to be more inhibitory than glucose which is also observed in β-galactosidase from other fungal sources [13, 20].

As shown in Table 6, the partially purified soluble β-galactosidase from *A. niger* UV-5 was found to exhibit 70% activity of the commercially used lactase

Table 4. Effect of metal ions on soluble β-galactosidase activity

Metal ion	% Activity at two metal ion concentrations, M		Metal ion	% Activity at two metal ion concentrations, M	
	1×10^{-3}	1×10^{-4}		1×10^{-3}	1×10^{-4}
$MnSO_4$	90	100	$MgSO_4$	100	100
$MnCl_2$	110	105	$FeSO_4$	100	110
NaCl	100	105	KCl	115	110
$CaCl_2$	80	90	EDTA (1-200 mM)	100	

* Activity with respect to β-galactosidase activity without addition of metal ions was taken as 100%.

Table 5. Effect of sugars on soluble β-galactosidase activity*

Concentration mM	Glucose	Galactose	Glucose + Galactose
0	100	100	100
20	100	22	33.3
50	94	17	19.7
100	84.8	13.3	17
200	78.8	10	14.3

* Expressed as % activity of the enzyme

Table 6. Comparison of commercial (*A. oryzae*) and soluble β-galactosidase from *A. niger* UV-5

Substrate	% Lactose hydrolyzed with Commercial enzyme A	B	Experimental enzyme A	B
Milk 30	25	20	25	
5% Lactose	85	80	60	80
Deproteinized Cheese whey	80	75	60	75

A - Incubated at 60°C for 6 hours
B - Incubated at 65°C for 6 hours

from *A. oryzae* at 60°C and equal activity at 65°C when used to hydrolyse lactose in the substrates viz; pure lactose solution, deproteinized cheese whey and whole milk. The diminution in the activity of commercial lactase at 65°C indicates its partial inactivation as a result of exposure to higher temperatures.

References

1. Heinrikson, R. L. and Tomasselli, A. G. Purification and characterization of recombinant proteins - opportunities and challenges. In *Purification and analysis of recombinant proteins*, (ed. R. Seetharam and S. K. Sharma) Marcel Dekker Inc. N. Y., Basel, Hong Kong. (1991).
2. Wendroff, W. L., Amundson, C. H., Olson, N. F. and Garver, J. C. Use of yeast β-galactosidase in milk and milk products. *J. Milk Food Technol.*, **34**, 294-299, (1971). Cited: *Chem. Abstr.*, **75**, 75030, (1971).
3. Cohn, M. and Monod, J. Purification and properties of the β-galactosidase (lactase) of *E. Coli*. *Biochem. Biophys. Acta.*, **7**, 153-159, (1951).
4. Ramana Rao, M. V. and Dutta, S. M. Purification and properties of β-galactosidase from *S. thermophilus*. *J. Fd. Sci.*, **46**, 1419-1423, (1981).
5. Nevalainen, K. M. H. Induction, isolation and characterization of *A. niger* mutant strains producing elevated levels of β-galactosidase. *Appl. Environ. Micro.*, **41**, (3), 593-596, (1981).
6. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**, 265-275, (1951).
7. Scopes, R. K. *Protein purification, principles and*

practice, (II edn). Springer-Verlag. (1987).
8. Nickerson, T. A., Vujicic, I. F. and Lin. A. Y. Colorimetric estimation of lactose and its hydrolytic products. *J. Dairy Sci.*, **56**, 386-390, (1976).
9. Bhal, O. P. and Agrawal, K. M. L. Glycosidases of *A. niger*. *J. Biol. Chem.*, **244**, 2970-2978, (1969).
10. Park, Y. K., DeSanti, M. S. S. and Pastore, G. M. Production and characterization of β-galactosidase from *A. oryzae*. *J. Food Sci.*, **44**, 100-103, (1979).
11. Greenberg, N. A. and Mahoney, R. R. Production and characterization of β-galactosidase from *S. thermophilus*. *Ibid.*, **47**, 1824-1828, (1982).
12. Mahoney, R. R., Nickerson, N. A. and Whitaker, J. R. Selection of strain, growth conditions, and extraction procedures for optimum production of lactase from *K. fragilis*. *J. Dairy Sci.*, **58**, 1620-1629, (1975).
13. Mahoney, R. R. and Whitaker, J. R. Purification and physicochemical properties of β-galactosidase from *K. fragilis*. *Ibid.*, **58**, 584-591, (1978).
14. Letunova, E. V., Tikhomirova, A. S., Shiyan, S. D. Markin, V. A., Khorlin, A. Ya. and Bezborodoc, A. M. Production and properties of β-galactosidase from *A. tenuis*. *Biokhimiya* (Moscow), (1981). Cited: *Chem. Abstr.*, **95**, 37883, (1981).
15. Roseanu, A., Lunga, M. and Vasu, S. S. Characterization of lactase produced by *A. niger*. *Stud. Cercet, Biochem.*, **25**, 47-52, (1982). Cited: *Chem. Abstr.*, **99**, 34964, (1983).
16. Roseanu, A. Lunga, M., Preda, N. and Vasu, S. S. Biochemical and biological characterization on *A. niger* lactase preparation. *Ibid.*, **27**, 92-96, (1984). Cited: *Chem. Abstr.*, **101**, 125606, (1984).
17. Letunova, E. V., Tikhomirova, A. S., Shiyan, S. D., Markin, V. A., Khorlin, A. Ya. and Bezborodov, A. M. Purification and properties of β-galactosidase. *Biokhimiya*, (Moscow), (1981). Cited: *Chem. Abstr.*, **95**, 37883, (1983).
18. Wierzbicki, L. E. and Kosikowski, F. V. Lactase potentials of various micro-organisms grown in whey. *J. Dairy Sci.*, **56**, 26-32, (1971).
19. Woychik, J. H. and Wondolowski, M. V. Lactose hydrolysis in milk and milk products by bound fungal β-galactosidase. *J. Milk and Fd. Technol.*, **36**, 31-33, (1973).
20. Borglum, G. B. and Sternberg, M. Z. Properties of a fungal lactase. *J. Fd. Sci.*, **37**, 619-623, (1972).
21. Ohitsuka, K., Tanoh, A., Ozawa, O., Kanematsu, T., Uchida, T. and Shinke, R. Purification and properties of a β-galactosidase with high galactosyl transfer activity from *C. laurentii* OKN-4. *J. Ferm. Biotech.*, **70**, 301-307, (1990).
22. Huffman, L. M., and Harper, W. J. Lactose hydrolysis in batch and hollow fibric membrane reactor. *J. Dairy Sci. Technol.*, **20**, 57-63, (1985).
23. Ueno, S., Miyama, A., Ohashi, Y. and Izumya, S. Production of thermoresistant β-galactosidase. *Jpn. Kokai Tokkyo Koho, JP*, **60**, (141), 288, (1985). Cited: *Chem. Abst.*, **104**, 18660, (1986).
24. Zeng, Y., Cheng, X. and Zhang, S. Purification and properties of β-galactosidase from *Aspergillus phoenicis*. *Shengisu Huaxue Zazhi*, **3**, (6), 552-560, (1987). Cited: *Chem. Abstr.*, **108**, 71162, (1988).
25. Gonzalez, R. R. and Monsan, P. Purification and some characteristics of β-galactosidase from *A. fonscecaeus*. *Enz. Microb. Technol.*, **13**, 349-352, (1991).
26. Griffiths, M. W. and Muir, D. D. Properties of a thermostable β-galactosidase from a thermophilic bacillus: Comparison of the enzyme activity of whole cells, purified enzyme and immobilized whole cells. *J. Sci. Fd. Agric.*, **29**, 753-761, (1978).
27. Giacin, J. R., Jakubowski, J., Gilbert, S. G., Leeder, J. G. and Kleyn, D. H. Characterization of lactase immobilized on collagen conversion of whey lactose by soluble and immobilized lactase. *J. Fd. Sci.*, **39**, 751-754, (1974).
28. Rahim, K. A. and Lee Byong, H. Specificity, inhibitory studies and oligosaccharide formation by β-galactosidase from psychrotrophic *Bacillus subtilis* RL 88. *J. Dairy Sci.*, **74**, (6), 1773-8, (1991).
29. Itoh, K., Toba, T., Itoh, T. and Adachi, S. Properties of β-galactosidase of *Lactobacillus Kefiranofaciens* K-1 isolated from kefir grains. *Lett. Appl. Microbiol.*, **15**, (5), 232-234, (1992).
30. Wendroff, W. L. and Amundson, C. H. Characterization of β-galactosidase from *S. fragilis*. *J. Milk Food Tech.*, **34**, 300-306, (1971).