JSR Publications

Available Online

J. Sci. Res. **4** (1), 239-249 (2012)

JOURNAL OF SCIENTIFIC RESEARCH

www.banglajol.info/index.php/JSR

Study on Isolation and Partial Purification of Lactase (β-Galactosidase) Enzyme from *Lactobacillus* Bacteria Isolated from Yogurt

N. H. M. R. Mozumder[1*], M. Akhtaruzzaman[2], M. A. Bakr[3] and Fatema-Tuj-Zohra[4]

[1]Department of Food Science and Nutrition, Hajee Mohammad Danesh Science and Technology University, Dinajpur-5200, Bangladesh

[2]Institute of Nutrition and Food Science, University of Dhaka, Dhaka-1000, Bangladesh

[3]Diet Solution Limited, Dhanmondi, Dhaka-1209, Bangladesh

[4]Nestle Nutrition Department, Nestle Bangladesh Limited, Dhaka, Bangladesh

Received 25 August 2011, accepted in revised form 22 November 2011

Abstract

Lactase has many applications in dairy industry including for the treatment of lactose intolerance. The present study was conducted to identify the activity of lactase enzyme produced by *Lactobacillus* bacteria isolated from yogurts available in Dhaka city. The strains were identified to be gram positive, catalase negative, fermentative and lactase producer when cultured on selective MRS agar media by using standard bacteriological procedures and techniques. The study revealed that enzymes produced by lactobacilli were capable to produce glucose from substrate lactose in lactose modified media using lactase assay Kit Glu IB and their highest protein concentration (17.25 mg/ml) was observed in the supernatant of culture media isolated from *L. lactis.* Highest total activity (850.69 U/l) and specific activity (50.04 U/mg) of lactase enzyme was observed in the strain of *L. bulgaricus*. The crude extract which showed highest activity was further purified by ammonium sulphate precipitation followed by anion exchange column chromatography (DEAE cellulose). Final specific activity and fold purification of lactase enzyme reached to 62.80 U/mg and 1.47 respectively. The highest physic-chemical properties (Effect of pH and temperature) of lactase enzyme were observed at P^H 6.0 which was 43.98 U/mg of protein and at 70°c temperature which was 111.11 U/mg of protein.

Keywords: β- Galactosidase; Specific activity; DEAE cellulose; Fold purification; Yogurt.

© 2012 JSR Publications. ISSN: 2070-0237 (Print); 2070-0245 (Online). All rights reserved.
doi:10.3329/jsr.v4i1.8478 J. Sci. Res. **4** (1), 239-249 (2012)

1. Introduction

The term lactose intolerance describes the inability to hydrolyze dietary lactose resulting from congenital or adult lactase deficiency, which is normally produced by the brush border of the small-intestinal mucosa [1, 2]. Approximately two-third people of the world

* *Corresponding author: rubel.infs@gmail.com*

are accounted to having the problem of lactose intolerance and it reduces significantly their quality of life [3]. This problem can be solved either by using supplementary probiotics microorganisms or using lactase enzyme in dairy products that hydrolyze the lactose present in food. But the use of microorganisms such as lactic acid bacteria as a probiotics is limited to treat the lactose intolerance due to it inability to form spore and endure the acidic environment of the stomach [4].

Lactase (β-Galactosidase; EC 3.2.1.2.3) enzyme hydrolyzes lactose, the main carbohydrate in milk, into glucose and galactose, which can be absorbed across the intestinal epithelium [5-7]. The importance of lactase is related to the use in milk and milk derivatives to decrease their lactose content solving the problem of low lactose solubility and its low degree of sweetening. Furthermore, the economic interest in this enzyme is related to its use in food and pharmaceuticals industries, because of the deficiency of lactase in many people [8]. The bacterial species currently used by the dairy industry which produced β-Galactosidase enzyme belong to genera of *Lactobacillus* and *Bifidobacterium* and comprise a limited collection of strains [9]. Although the yeast *Kluyveromyces lactis* is still the major commercial sources of lactase because of their dairy environmental habitat but its major drawback is its lower thermo stability [10]. But lactobacilli isolated from fermented Ragi produced lactase enzyme in significant quantities which is thermostable and prevent microbial contamination in milk processing [11]. Thus, this enzyme has considerable industrial potential because they can give better yields at high temperatures [12].

Though several studies on microbial lactase have been carried out throughout the world, there appear few studies on lactase enzyme produced by lactobacilli isolated from fermented milk product in Bangladesh. Hence an attempt has been made for the production of lactase enzyme from yogurt since yogurt is one of the popular fermented milk products which are a sub continental equivalent of curd. For its preparation the milk is allowed to ferment with the help of the particular species of lactic acid bacteria starter such as strains of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. The aim of present study was to isolate and partially purify the lactase enzyme from lactobacilli isolated from yogurt available in Bangladesh. The preparation of enzyme from lactobacilli strains was done by separation technique and its activity was measured according to the method as described by Hans Ulrich Bargmeyer [13]. The enzyme produced from this bacterium may be used in dairy industry for the development of lactose intolerance.

2. Materials and Methods

2.1. *Chemicals, reagents and bacteriological media*

The Bovine Serum Albumin (BSA), Folin Ciocalteau Reagent (E. Merck Germany), MRS (Hi Media Laboratory Pvt. Ltd, India) were purchased and collected from local scientific store except Glu IB kit (Wako Japan) and DEAE cellulose (Wako Japan). All other reagents such as maleate buffer, lactose solution, Tris buffer and glucose

standard solution were available in the laboratory of institute of Nutrition and Food Science, Dhaka University.

2.2. ***Sampling and analysis of organolaptic characteristics of yogurt***

A total number of ten traditionally manufactured popular yogurt samples (JGS: Jadab Gosh and Sons, BS: Bikrampur Sweets, CM: Commilla Mistanno Bhander, MS: Muslim Sweets, AD: Aftab Dhai, TMG: Tripty Misty, RM: Rajshahi Misty Ghar, HD: Haque Dhai, BD: Bogra Dhai Bhander, MCG: Mohon Chan & Grand Sons) were collected from different sweetmeat shops of Dhaka City and their organolaptic characters viz., color flavor, consistency taste and pH were analyzed through sensory evaluation.

2.3. ***Isolation and identification of lactobacillus bacteria***

The isolation of genus of Lactobacillus sp. was done on de Man Rogosa and Sharrp agar (MRS agar, Hi-media, M-369) [14]. Clear and discrete colonies of presumptive *Lactobacillus* that isolated by repeated pour plate and spread plate techniques using selective M.R.S. agar at pH of 6.2-6.6 were used for pure culture and preserved in refrigerator at 4-7ºC for sub culturing. The presumptive lactobacilli isolates were characterized and identified on the basis of cultural growth (shape, edge, elevation, opacity, consistency), cellular morphology (gram reaction, motility, shape) biochemical (catalase test, acid production test from different sugars, NH_3 production from Arginine for, CO_2 gas from glucose in Gibson's semi solid Tomato Juice Medium) and physiological characteristics; growth at different temperature and pH [15].

2.4. ***Enzyme preparation***

For the production of lactase, presumptive lactobacilli was grown in MRS broth (100ml) with 0.1 ml of culture inoculums and incubated at 37°C for 24- 48 hours. After incubation cells were removed by centrifugation at 12,000X g RPM for 20 minutes (Kokusan-H-200NR centrifuge machine, Japan) below the temperature of 4ºC. The cell free supernatants (crude enzyme) containing intracellular materials were stored in the freezer and used for enzymatic assay and partial purification.

2.5. ***Determination of concentration of extracellular soluble protein***

Protein concentration was determined with the method of Lowry *et al.,* [16] using Bovine Serum Albumin (BSA) as the standard and optical density of the reaction mixture was measured at 660nm by the spectrophotometer. The amount of the soluble protein was calculated from the standard curve as mg of protein per ml of test samples.

2.6. ***Assay of lactase enzyme: catalytic and specific activity determination***

The lactase activity was measured as a total lactase [13] by incubating with lactose followed by the measurement of the glucose liberated with a glucose oxidase reagent (GOD> 2400 U/L, POD 1100 U/L) served from Glu IB kit (Wako Japan) providing glucose oxidase , peroxidase and buffer. A 20µl of lactose solution (56m.mol/l) in maleic acid was mixed with 40µl of supernatant and incubated at 37ºC for 60 minutes and finally added with 3ml of glucose reagent and again incubated for 30 minutes and liberated glucose was measured by spectro-photometrically at 510 nm against blank.

2.6.1. *Calculation*

The amount of glucose formed (colored substances) per unit time is measured from the standard curve. One unit of enzyme activity was defined as the amount of enzyme hydrolyzing 1 micromole (µmole) of substrate lactose sugar per minute at 37°c and was expressed in units per milligram or enzyme protein (Eqs. 1, 2 and 3).

Specific activity (U/mg of protein) = Enzyme activity/protein concentration (1)
Purification fold = Specific activity in the collected phase/initial specific activity (2)
Recovery (%) = enzyme activity of the phase/ total enzyme activity added to the system (3)

2.7. ***Partial purification of lactase enzyme***

For this purpose, the clarified supernatant obtained from first centrifugation of culture media brought to 80% ammonium sulfate precipitation in a refrigerated condition and dialyzed by dissolving in 10mM Tris- HCL (pH 7.0) buffer for 24 hours and finally centrifuged at 15000 g X rpm for 20 minute (Kokusan-H-200NR centrifuge machine, Japan). All subsequent steps involving ammonium sulfate were carried out at 4°c. The obtained pellet was further purified by an ion exchange column chromatography. An ion exchange chromatography was performed on column using a DEAE cellulose bed and equilibrated with buffer 10mM Tris-HCL; the target enzyme was eluted by a linear gradient of 250mM NaCl in 10 mM Tris-HCl buffer. The fractions which showed highest activity were pooled for lactase activity at different pH and temperatures.

2.8. ***Effect of pH and temperature on enzyme activity***

The effect of pH on lactase enzyme was determined at optimum temperature (37^{0}c) in five buffer system by incubating the enzyme in water bath for 1 hour and finally by the measurement of glucose liberated with a glucose standard reagent (Glu IB Kit) as described by earlier study[13].

1. 0.2M KCl-0.2M HCL buffer (Clark & Lubs' solution)- pH 1.0 and 2.0
2. 0.1M Citric acid-0.2 M Na_2HPO_4 buffer - pH 3.0,4.0 and 5.0
3. 0.1M Citric acid-0.1M Tri-sodium citrate buffer- pH 6.0 and 7.0
4. 0.2M Glycine-0.2M NaOH buffer solution- pH 8.0 and 9.0
5. 0.1M Sodium carbonate-0.1M Sodium bicarbonate buffer solution- P^H 10.0

The effect of temperature on enzyme activity was determined at temperatures ranges 10 to 70°c by incubating in water bath with constant agitation and aliquots were withdrawn after 1 hour and specific activity was determined as described by earlier [13].

3. Results and Discussion

The present study focuses on the isolation and partial purification of lactase enzyme from *Lactobacillus bacteria* isolated from fermented milk product such as yogurt. *Lactobacilli* are the most abundant bacteria in gastrointestinal tract and produce lactase enzyme in dairy products. Though, the best sources for commercial lactase enzyme are microbial enzymes such as *Escherichia coli*, *Aspergillus niger,* some strains of lactose-fermenting yeast and bacteria, and recently a mutant strain of *A. foetidus*, *Lactobacilli* bacteria have become an object of interest for the commercial production of lactase enzyme for the treatment of lactose intolerance [7, 17, 18].

Table 1. Organoleptic characteristics of yogurts.

Sample	Product	Source[1]	Taste	Flavor	Color	Consistency	Why*(+/-)*	P^H
1	Yogurt	JGS	Sweet	Characteristic	Brownish	Semisolid	-	3
2	Yogurt	BS	Sweet	Characteristic	Yellowish	Semisolid	+	4
3	Yogurt	CM	Sweet	Light flavor	Yellowish brown	Semisolid	-	4
4	Yogurt	MS	Sweet	Characteristic	Light brown	Semisolid	-	3
5	Yogurt	AD	Sweet	Curd flavor	Brown	Semisolid	+	3
6	Yogurt	TMG	Sweet	Characteristic	Yellow	Semisolid	+	4
7	Yogurt	RM	Sweet	Curd flavor	Brownish	Semisolid	-	3
8	Yogurt	HD	Sweet	Characteristic	Brownish	Semisolid	-	4
9	Yogurt	BD	Sweet	Light flavor	Yellow	Semisolid	-	4
10	Yogurt	MCGS	Sweet	Light flavor	Light brown	Semisolid	-	3

[1]JGS: Jadab Gosh and Sons, BS: Bikrampur Sweets, CM: Commilla Mistanno Bhander, MS: Muslim Sweets, AD: Aftab Dhai, TM: Tripty Misty, RMG: Rajshahi Misty Ghar, HD: Haque Dhai, BD: Bogra Dhai Bhander, MCG: Mohon Chan & Grand Sons.

Table 1 shows the organolaptic characteristics of ten yogurts such as, taste, color, flavor, consistency, whey presence (+) or absence (-) and pH by sensory evaluation. The entire samples were sweet and semi-solid in consistency. The pH ranges recorded for all samples were 3.0 – 4.0. The presence of whey observed only in three samples i.e., Bikrampur sweets (BS), Aftab Dhai (AD) and Tripty Mishti ghar (TMG). Maximum samples had characteristic curd flavor and yellowish in colors.

Table 2. Isolation and identification of *Lactobacilli* by different tests.

Tests			Sources of strains									
			JGS	BS	CM	MS	AD	TMG	RM	HD	BD	MCGS
Gram Staining			(+ve) rod	(+ve) rod	(+ve) rod	(+ve) rod	(+ve) rod	(+ve) rod	(+ve) rod	(+ve) rod	(+ve) rod	(+ve) rod
Wet mount			Motile	Motile	Motile	Motile	Motile	Motile	Motile	Motile	Motile	Motile
Catalase test			-	-	-	-	-	-	-	-	-	-
NH3 from arginine			-	+	-	-	-	+	-	+	+	-
CO_2 from Gibson's semi solid media			-	-	-	+	+	-	-	-	-	-
Temperature(^{0}C)	10		-	-	-	-	-	-	-	-	-	-
	20		-	-	-	-	-	-	-	-	-	-
	30		++	++	-	++	++	++	++	++	++	++
	35		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	37		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	38		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	45		+++	++	++	++	+++	+++	+++	++	+	+++
pH	4.5		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	6.5		++	++	++	++	++	++	++	++	++	++
	8.5		-	-	-	-	+	-	-	+	-	+
Sugar fermentation	Lactose	A[1]	+	-	-	+	-	+	+	+	+	+
		G[2]	-	-	+	-	+	-	-	-	-	-
	Sucrose	A	+	-	-	+	+	+	+	+	+	+
		G	-	+	-	-	+	+	-	-	-	-
	Raffinose	A	+	+	+	+	+	+	+	+	-	+
		G	-	+	-	-	+	+	-	-	-	-
	Arabinose	A	-	-	-	-	-	-	-	-	-	-
		G		-	-	-	-	+	-	-	-	-
	Glucose	A	+	+	+	+	+	+	+	+	+	+
		G	-	-	-	-	-	-	-	--	-	
	Salicin	A	+	-	-	+	-	+	+	+	+	+
		G	-	-	-	-	+	+	-	-	-	-
	Fructose	A	-	+	-	-	-	+	+	-	+	+
		G	-	-	-	-	-	-	-	--	-	-
Resembled sp.			*L. lactis*	*L. brevis*	*L. bulgaricus*	*L. lactis*	*L. delbrueckii*	*L. acidophilus*	*L. lactis*	*L. bulgaricus*	*L. lactis*	*L. bulgaricus*

[1]A = Acid production detected by color change, [2]G = Gas production detected in Durham's tube, [3](+) = positive result, [4](-) = negative result, [5]+ve = positive, [6]+++ = highest growth, ++ = moderate growth, + = lowest growth.

The fastidious nature of Lactobacilli requires rich medium for their growth and production of lactase enzyme. So, the pure cultures of the isolated strains were

successfully obtained which had abilities to ferment lactose by growing them in lactose modified MRS broth and then on agar surface through streaking. The cellular morphological, physiological, fermentation and common biochemical characteristics of the isolates were shown in Table 2. During isolation the generic identity of the genera was considered as presumptive *Lactobacillus.* Gram positive, long slender rods to short rod bacilli type in chains, motile, catalase negative characteristics were observed in all samples.

From the Table 2 it can be shown that strains grew very well at pH 4.5. Acid and gas production (fermentation test) from sugars were observed in Durham's tube using selective media modified with sugars. The identities of the strains were confirmed after comparing the characters of the strains of *Lactobacillus bulgaricus*, *L. brevis, L. acidophilus, L. delbrueckii, and Lactobacillus lactis* as described in Bergey's manual of determinative bacteriology [19] though they were differed by minor characters such as NH_3 production from arginine, growth at 45^0c and acid from different sugars. However, Jiang *et al.* [20] identified and characterized the microbial lactase and reported that lactic acid bacteria including *Lactobacillus acidophilus* and *L. bifidus* have appreciable lactase activity.

Table 3 summarizes the results of supernatant fractions, protein concentration of cell free supernatant, concentration of glucose produced during lactase assay using glucose standard curve, total activity (catalytic activity) and specific activity of lactase that was assessed on supernatant fractions by separating the culture suspension. From Table 3, it is shown that the total activity in the range of 11.57- 512.57 (U/l) and specific activity in the range of 0.826-50.04 (U/mg) of protein was observed in all the strains and the enzyme isolated from strain *L. bulgaricus* showed the highest lactase activity, 50.04 (U/mg) of protein with highest protein concentration and glucose liberation during assay whereas lowest specific activity, 0.826 (U/mg) of protein was observed in strain *L. delbrueckii*.

Table 3. Summary of lactase activity from different *Lactobacillus* bacteria.

Sources of Strains	*Lactobacillus sp.*	*Supernatant fractions (ml)*	*Protein conc. (mg/ml)*	*Glucose conc. (μg/ml)*	*Total activity (U/l)*	*Specific activity (U/mg)*
JGS	*L. lactis*	78	12.00	221.25	512.15	42.67
CM	*L. bulgaricus*	64	17.00	367.50	850.69	50.04
AD	*L. delbrueckii*	64	14.50	5.00	11.57	0.826
RM	*L. lactis*	65	17.25	131.25	303.819	17.612
MCS	*L. bulgaricus*	78	9.00	86.25	199.65	22.18

*Source: JGS: Jadab Gosh and Sons, CM: Commilla Mistanno Bhander, AD: Aftab Dhai, RM: Rajshahi Misty Ghar, MCG: Mohon Chan & Grand Sons.

It is known that lactase is an intracellular enzyme, primarily bound to cell walls [21]. The total activity of lactase in the range of 800-1000 U/ml for whole cell suspension and 250-300U/ml for intracellular extract was reported [12]. The range of specific activity of the lactase enzyme (20-79 U/mg of protein; Mean ± SD = 40.0 ± 15.6) was demonstrated that is a good agreement with our highest value (50.04 U/mg of protein) obtained from *L. bulgaricus* [22]. Moreover, higher value of β-Galactosidase enzyme in commercial strains of *L. delbrueckii* isolated from cheese was also reported [23]. So, the enzyme produced from *L. delbrueckii subsp. bulgaricus* strain further emphasize the importance of selecting appropriate strains for use in industrial production of lactase.

For the purification of lactase, the crude extract of lactase obtained after centrifugation from the strain of *L. lactis* was further purified using ammonium sulfate precipitation, centrifugation and DEAE cellulose chromatography. The lactase activity was eluted as a two way-symmetrical peak at 250 mM NaCl in10 mM Tris-HCl buffer in Fig. 1.

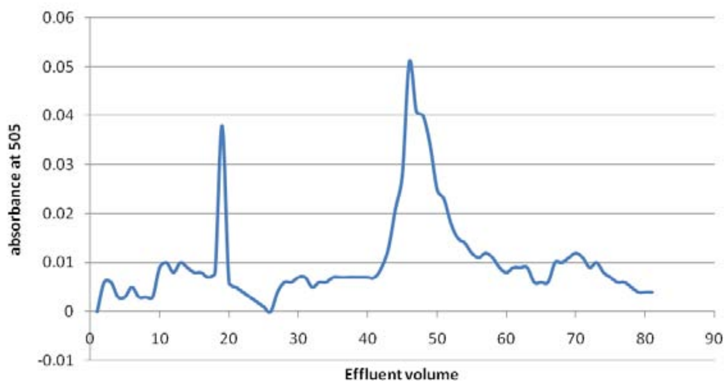

Fig. 1. Ion exchange (DEAE cellulose) chromatography of lactase.

The purification process and the results of the lactase enzyme are summarized in Table 4, which depicts specific activity and the purification fold of every step. Purification is very important for enzyme production. Fractions volume (elution pattern) collected from chromatography showed a symmetrical peak consistent with previous report [24]. Pastore and Park, 1981 purified β-Galactosidase from *Scopulariopsis spp* by precipitation with ammonium sulphate and two chromatographic steps leading to a 4% yield of the pure enzyme desired. The purification factor increased 1.6-fold and the recovery of the target enzyme was 83% [25]. In our study an overall recovery (% yield) of 0.306 and a 1.47 purification fold enrichment of the enzyme were obtained after 3 steps. The final specific activity reached 62.8 U/ mg of protein and compared with the values reported by the previous studies [7, 11, 26, 27].

Table 4. Summary of the lactase purification from *Lactobacillus lactis.*

Strain name	Purification Steps	Fraction volume (ml)	Protein conc. (mg/ml)	Catalytic activity (U/l)	Sp. activity (U/mg)	Field (%)	Purification fold
L. lactis	Cell free supernatant	78	12.00	512.15	42.67	100	1.0
	80% ammonium sulfate precipitation & dialysis	25	1.5	83.43	55.62	16.29	1.30
	ion exchange (DEAE) (pooled fractions)	5	0.025	1.57	62.80	0.306	1.47

Physico-chemical properties (pH and temperature) played an important role for the production of enzyme. The pH profile of the lactase activity after purification is shown in Fig. 2. The enzyme displayed its maximum activity at pH 6.0 with little activity outside pH 3.0 and pH 9.0.The purified thermostable β-Galactosidase isolated from *Bacillus stearothermophilus* in milk showed maximum activity at pH 7.0 which is in a good agreement with our result [11]. It may be noted that different pH optimum are the result of the presence of several enzymes in crude preparation or by the presence of several substrates on which the enzyme can act.

The effect of temperature on the enzyme activity was studied at temperatures ranging from 30 to 80°C at pH 7.0 (Fig. 3). Thermo stability of lactase is of great important for the economy of their food consumption and food industrial application. The effect of temperature on lactase was also observed and it was found that with the increase of temperature activity was gradually increased. It has been established that for many enzymes, which are useful in food processing becomes appreciable at temperature above 45°C [28]. Chen *et al.,* 2008 in their study showed that the thermophilic enzyme β-Galactosidase performed best activity at 70°C temperature. Our result was in line with good consistent as reported by the study [11].

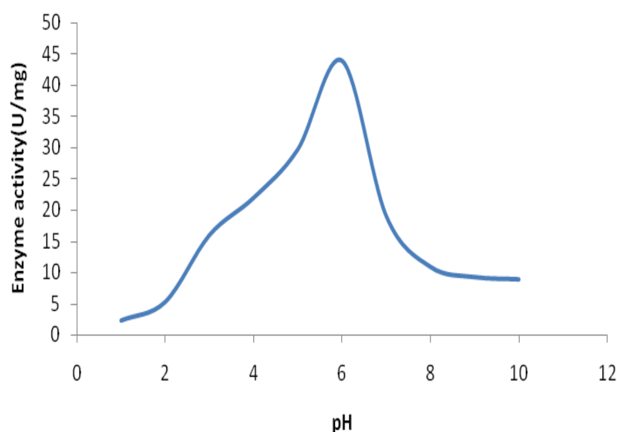

Fig. 2. Specific activity of lactase enzyme at various pH.

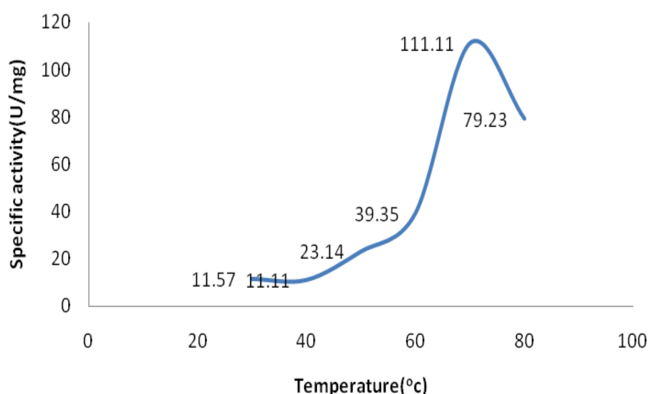

Fig. 3. Specific activity of lactase enzyme at various temperatures.

4. Conclusion

This paper reports the isolation and partial-purification of lactase enzyme from *Lactobacillus* strains isolated from yogurt. Once the strains had been isolated it was characterized by various tests and screened for efficient lactase enzyme producer. The enzyme is active at high temperature up to 70°C and showed highest activity at pH 6.0 which indicates its high level of thermostability. The maximum total activity of enzyme was recorded as 850.69U/l. The purification fold was 1.47 after column chromatography. After considering the all findings it might be concluded that the enzyme produced in this work was lactase that showed a consistent with results obtained from previous study.

Acknowledgement

We acknowledge the financial support (Fellowship, 2008) from the Ministry of Science, Information and Communication Technology of Bangladesh. We also thank the curator of Microbiology Laboratory, Institute of Nutrition and Food Science and faculties of Botany Department, Dhaka University.

References

1. M. D. Vrese and A. Stegelmann, Am. J. of Clin. Nutr. **73**, 421 (2001).
2. P. Karasova, V. Spiwok, S. Mala, B. Kralova, and N. Russell, Czech J. Food Sci. **20**, 43 (2002).
3. B. A. Pribila, S. R. Hertzler, and B.R. Martin, J. Am. Diet. Asso. **100** (5), 524 (2000). http://dx.doi.org/10.1016/S0002-8223(00)00162-0
4. M. Begley, C. Hill, and G. M. Cormac, J. of Appl. Environ. Microbiol. **72** (3), 1729 (2006). http://dx.doi.org/10.1128/AEM.72.3.1729-1738.2006
5. T. Vasiljevic, and P. Jelen, J. Innov, Food Sci. Emerg. Technol. **2**, 75 (2001). http://dx.doi.org/10.1016/S1466-8564(01)00027-3
6. J. T. Troelsen, J. Biochimica. Biophysica Acta. **1723**, 19 (2005). http://dx.doi.org/10.1016/j.bbagen.2005.02.003

7. M. B. Heyman, J. Pediatc. **118,** 1297 (2006).
8. J. E. Prenosil, E. Stuker, and J. R. Bourne, Biotechnol. Bioeng. **30,** 1026 (1983). http://dx.doi.org/10.1002/bit.260300905
9. T. He, M.G. Priebe, Y. Zhong, C. Huang, H. J. Harmsen, G. C. Raangs, J. M. Antoine, G.W. Welling, and R.J. Vonk, J. Appl. Microbial. **104** (2), 595 (2008). PMid:17927751
10. V. B. Ganeva, Galutzov, and N. Eynard, Appl. Microbial. Biotechnol. **56**, 411 (2001). http://dx.doi.org/10.1007/s002530100642
11. W. Chen, H. Chen, Y. Xia, J. Zhao, F. Tian, and H. Zhang, J. Dairy Sci. **91**, 1751 (2008). http://dx.doi.org/10.3168/jds.2007-617
12. S. K. Akolkar, A. Sajgure, and S. S. Lele, Ind. J. Biotechnol. **5**, 184 (2006).
13. H. U. Bargmayer, Method of Enzymatic Analysis, 3rd edition, Vol. 2 (Verlag Chemie, 1985) pp. 208-216, 227-230.
14. J. C. Man, M. Rogosa, and M. E. Sharpe, J. of Appl. Bacteriol. **23,** 130 (1960). http://dx.doi.org/10.1111/j.1365-2672.1960.tb00188.x
15. W. F. Harrigan, and M. E. McCance, Laboratory Manual of Food and Diary Microbiology (Academic Press, California, 1998).
16. O. H. Lowery, N. J. Rosebrough, A.L. Farr, and R.J. Randall, J. Biol. Chem. **193**, 265 (1951). PMid:14907713
17. I. Petzelbauer, B. Nidetzky, D. Haltrich, and K.D. Kulbe, Biotechnol. Bioeng. **64**, 322 (1999). http://dx.doi.org/10.1002/(SICI)1097-0290(19990805)64:3<322::AID-BIT8>3.0.CO;2-9
18. R. Tabasco, T. Paarup, C. Janer, C. Pelaez, and T. Requena, Int. Dairy J. **17,** 1107 (2007). http://dx.doi.org/10.1016/j.idairyj.2007.01.010
19. R. E. Buchanan, and N. E. Gibbons, Bergey's Manual of determinative Bacteriology, 8th ed. (Williams & Wilkings, Baltimore, 1974) pp. 579-583.
20. T. Jiang, A. Mustapha, and D. A. Savaiano, J. Dairy Sci. **80** (8), 1537 (1997). http://dx.doi.org/10.3168/jds.S0022-0302(97)76083-1
21. G. Montanari, C. Zambonelli, L. Grazia, M. Benevelli, and C. Chaivari, J. Food Technol. Biotechnol. **38,** 129 (2000).
22. A. Dahlqvist, Int. Review of Biochem. (Biochemistry of carbohydrate-II), Vol. 16 (University Park Press, Baltimore, 1978).
23. C. G. Vinderola and J. A. Reinheimer, J. Food Res. Int. **36**, 895 (2003). http://dx.doi.org/10.1016/S0963-9969(03)00098-X
24. H. Skovbjerg, H. Sjosirom, and O. Noren, Eur. J. Biochem. **114,** 653 (1981). http://dx.doi.org/10.1111/j.1432-1033.1981.tb05193.x
25. G. M. Pastore and Y. K Park, J. Ferm. Technol. **58** (1), 79 (1981).
26. M. E. Silva, and T. T. Franco, Rev. de Microbiol. **30**, 324 (199).
27. G. Rajaram, P. Manivasagan, B. Thilagavathi, and A. Saravanakumar, Adv. J. Food Sci. Technol, **2** (2), 137 (2010).
28. G. Reed, Enzymes, *In*: Food Processing, 2nd ed. (Academic Press, London, 1975) pp. 2-97, 383-389 & 172-174.