

Characterization of β -Galactosidase from Lactose Utilizing Yeast Isolated from the Dairy Sample

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ABSTRACT

Objectives: The objective of the study was to isolate lactose positive yeasts from dairy samples collected from local markets of Kathmandu, to extract crude β -galactosidase from the lactose positive yeast and to characterize the enzyme for optimum time duration, pH, temperature, Michaelis-Menten constant (K_m) and maximum activity (V_{max}).

Methods: Four lactose positive yeast strains were isolated from dairy samples collected from local market of Kathmandu by pour plate method. Single strain having maximum lactose positive activity was selected for the study. The mass culture of the lactose positive yeast strain was lysed by 2% Chloroform and the yeast cell lysate containing β -galactosidase (i.e. crude enzyme extract) was characterized by using ONPG (Ortho-Nitrophenyl- β -D-galactopyranoside) as substrate. ONPG is a colorless substrate for the enzyme assay which is hydrolyzed by the enzyme into yellow colored product ONP (Ortho-Nitrophenol). The concentration of product formed was monitored spectrophotometrically at 420 nm to determine the enzyme activity and to characterize the enzyme.

Results: The enzyme had wide range of working temperature from 0-50°C, with optimal temperature of 37°C. However, greater than 50% hydrolyzing ability was maintained in the range of 14-40°C. Optimum time of reaction was 70 min. The enzyme had maximum activity in the near neutral pH of 6.8. Michaelis-Menten constant of the enzyme was found to be 2.23 mM of ONPG and V_{max} was 58.82 nmol/min/ml. Enzyme activity was 27.88 nmol/min/ml, Specific enzyme activity was 59.97 nmol/min/mg and total enzyme activity was 3346.33 nmol/min.

Conclusion: The activity over a wide range of temperature 0-50°C with low K_m value shows that the enzyme has a commercial application in clearance of lactose pollution in waste water in different environmental conditions.

Keywords: Yeast, β -galactosidase, enzyme activity, ONPG, K_m

INTRODUCTION

β -galactosidase (E.C.3.2.2.1.23), also called beta-gal or β -gal, is a hydrolase enzyme that catalyses hydrolysis of beta-galactosides into monosaccharides. It is an exo-glycosidase which hydrolyses the β -glycosidic bond formed between a galactose and its organic moiety. Substrates for different β -galactosidases include ganglioside GM1, lactosylceramides, lactose and

various glycoproteins. It may also cleave fucosides and arabinosides with much lower efficiency. β -galactosidase catalyzes the hydrolysis of lactose, abundant disaccharide in milk to monosaccharides; glucose and galactose (Husain 2010). β -galactosidase hydrolyses the β -1,4-D-galactosidic linkage of lactose, as well as those of related chromogens, O-nitro-phenyl- β -D-galactopyranoside (oNPG), p-nitrophenyl- β -D

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galactopyranoside (PNPG) and 6-bromo-2-naphthyl-galacto pyranoside (BNG) (Mahoney 1998).

Glycoside hydrolases (GH_s) including β -galactosidase constitute 113 protein families (Cantarel et al. 2009). Lactose degrading β -galactosidases (lactases) are classified as GH2 family and others are classified as GH42 family which shows activity towards β -galactosyl linkages in oligosaccharides (Hinz et al. 2004; Shipkowski et al. 2006). Lactase is often confused as an alternative name for β -galactosidase, but it is actually simply a sub-class of β -galactosidase.

Some β -galactosidases may also have an activity of transferring one or more, D-galactosyl units onto lactose (Rabiu et al. 2001). This enzymatic property is called transgalactosylation which is potential for the production of GOS (Galacto-oligosaccharides) (Miller and Whistler 2000).

β -galactosidase is an important enzyme in food, pharmaceutical industries and also in waste management. β -galactosidase can be used to avoid problems of lactose intolerance in individuals who are deficient in lactase. Lactose intolerance is a common problem prevalent in more than half of the world's population (Amir and Whorwell 2009; Johnson et al. 1993; Suarez and Savaiano 1997). High lactose content in non-fermented, sweetened, condensed and frozen dairy products such as ice-cream and condensed milk can lead to excessive lactose crystallization resulting in products with mealy, sandy or gritty texture (El-Kader et al. 2012). Thus β -galactosidase can be used to avoid the crystallization of lactose in sweetened, condensed and frozen dairy products. This helps to improve sweetness and to increase the solubility of milk products (Kara, 2004). It can be used to manage biodegradable wastes of food, dairy and beverage industries. Lactose is one of the major components of whey in cheese industry that is non-friendly in environment. Whey is the relatively clear supernatant that remains after the coagulated casein is separated from the milk for cheese making. Its lactose content is 4.5 to 5% (Nahvi and Moeini 2004). GOS are non-digestible oligosaccharides comprised of 2-20 molecules of galactose and 1 molecule of glucose (Miller and Whistler 2000). GOS are recognized as prebiotics since, they can stimulate the proliferation of lactic acid bacteria and *Bifidobacteria* in human intestine (Sako et al. 1999) [15]. So, much attention has been given to the production of GOS, especially *via* enzymatic

transgalactosylation, since the chemical synthesis is very tedious (Sears and Wong 2001).

β -galactosidases are widely distributed in numerous biological systems e.g. Plant and animal tissues, microorganisms (Fungi-yeasts and molds, actinomycetes) (Zadow 1992). Compared to the animal and plant sources of the enzyme, microorganisms produce enzyme at higher yields and thus lower the price of commercial β -galactosidase production (Santos et al. 1998). Thus, for large scale commercial production of the enzyme much work is focused on microbial production. The characteristics of the enzyme vary depending on its source. In general, β -galactosidase from yeasts and bacteria have optimum pH near neutral range (6.5-7) whereas the enzyme from molds act better at acidic range (3-5) (Wierzbicki and Kosikowski 1973).

The most studied β -galactosidase is the one produced by *Escherichia coli* but due to possible toxic factor associated with the coliform, the crude isolate of β -galactosidase is unlikely to be used in food processes (Santos et al. 1998). Therefore, β -galactosidases used in industrial scale for the production of milk and dairy products are isolated from microorganisms with GRAS status (generally recognized as safe). The commercial β -galactosidase are generally extracted from yeasts (*K. lactis* and *K. fragalis*) and fungi (*Aspergillus niger* and *A. oryzae*) (Harju 1987).

So far, no studies have been made on β -galactosidase extracted from lactose utilizing yeasts isolated from dairy samples in Nepal. Hence, we aimed to characterize β -galactosidase extracted from lactose utilizing yeasts isolated from dairy samples collected from local market of Kathmandu valley and evaluated the potential applications of enzymes in clearance of lactose pollution in waste water.

MATERIALS AND METHODS

Isolation: Eight different dairy samples were collected from different dairies in Kathmandu valley (4 yoghurt samples, 2 milk samples and 2 cotton cheese/farmer cheeses/paneer samples). 1gm of each dairy sample was taken for serial dilution upto 10^{-5} . 100 μ l of dilutions (10^{-2} 10^{-4} , 10^{-5}) were spread on YGCA Plates (5 gm Yeast extract, 20 gm Glucose, 0.1 gm Chloramphenicol in 1 liter of distilled water) and incubated at 25°C for 72 hrs. Yeast colonies with distinct morphological differences were selected and then purified by streaking on PDA plates and stored at 4°C (Nahvi and Moeini 2004).

Sugar assimilation test: Sugars used were Glucose, Fructose, Galactose, Sucrose, Lactose, Maltose, Raffinose, Xylose, Mannitol, and Dextrose. Sugar assimilation test was done using basal fermentative medium YEP Broth containing chloramphenicol (4.5 gm Yeast extract, 7.5 gm Peptone, 20 gm sugar (Raffinose 40 gm), Chloramphenicol 0.1 gm per liter). The final working solution was made by addition of 4 ml of Bromothymol blue stock solution (50 mg/75 ml in distilled water) to 100 ml of basal fermentative medium. After sterilization by autoclaving, chloramphenicol was added to the basal media. 10 ml of basal media was dispensed into sterile test tubes and test tubes were inoculated with yeast isolates and incubated at 25°C for 72 hrs. Test tubes were inspected at frequent intervals and positive sugar assimilation results were indicated by change in color of indicator from green to yellow. Control tubes were used in sets to monitor contamination. Lactose positive yeast isolates were identified from this test and these isolates were subjected to further study and mass culture (Nahvi and Moeini 2004).

Study of colonial and microscopic morphology:

Colonial morphology of lactose positive yeast isolates on PDA plates such as color, pigmentation, consistency, elevation, shape, size, margin, opacity was studied. The microscopic morphology of the lactose positive yeast isolates was studied after gram staining (Barnett et al. 1990; Nahvi and Moeini 2004).

Mass culture: Mass culture of lactose positive yeast isolates was done in enrichment media YEP broth containing chloramphenicol (10 gm Yeast extract, 20 gm Peptone, 20 gm Lactose, 0.1 gm Chloramphenicol per liter of distilled water). Yeast isolates were inoculated into 1000ml of sterile broth (pH 7.0) in sterile condition in a conical flask. The flask was then incubated in water bath shaker at 30°C for 48 hours (Nahvi and Moeini 2004).

Enzyme extraction: 1000 ml YEP broth was centrifuged at 7000 rpm for 10 minutes and resulting pellet of yeast cells was first washed with 20 ml phosphate buffer (pH 7.0, 0.1M) and then re-suspended in 120 ml of phosphate buffer (pH 7.0, 0.1M). Chloroform (2% v/v) was added to lyse the yeast cells and incubated overnight at 37°C. Yeast lysate was then centrifuged at 10,000 rpm for 20 minutes. Supernatant was then collected. Supernatant is the crude enzyme extract (Nahvi and Moeini 2004).

Determination of protein concentration: Protein concentration was determined by Bradford method. In 6 test tubes working BSA solution (1mg/ml) 10, 20, 40, 60, 80, 100µl was taken respectively. Volume was maintained to 200µl by addition of PO₄ buffer (pH 7.0, 0.01M). 2300 µl of Bradford reagent was added to each test-tubes and the final volume was 2500µl. Absorbance was taken at 595 nm against the blank (200 µl PO₄ buffer and 2300µl Bradford reagent) after 5min of incubation. Calibration curve was made for protein concentration Vs. absorbance. 40µl of test sample (β-galactosidase extract) was taken in a test tube and volume was maintained to 200µl by addition of PO₄ buffer. 2300 µl of Bradford reagent was added and after 5min of incubation absorbance was taken at 595 nm. The concentration of the test sample was determined by comparing the absorbance of the sample with the calibration curve (Bradford 1976).

Preparation of enzyme substrate: (ONPG) Ortho-Nitrophenyl-β-D-galactopyranoside (0.01 molesL⁻¹) prepared in a phosphate buffer of pH 6.8 was used as substrate for the β-galactosidase assay (Zhou and Chen 2001).

Determination of enzyme activity: 750µl of substrate solution (ONPG) with pH 6.8 and 250 µl of crude enzyme extract were added in a test tube. 2ml of phosphate buffer (pH 6.8, 0.1 M) was added to the test tube and then incubated at 37°C for 70 minutes. The reaction was quenched by adding 250 µl Na₂CO₃ (0.5 moles L⁻¹). Reaction progress was determined spectrophotometrically at 420 nm against the blank (750 µl ONPG, 2250 µl phosphate buffer of pH 6.8) (Fernandes et al. 2002).

Enzyme activity was calculated by accordingly.

$$\text{Enzyme activity} = \frac{0D420 \times \text{Reaction Volume}}{0.0045 \times \text{Enzyme volume} \times \text{Time}}$$

$$\text{Specific enzyme activity} = \frac{0D420 \times \text{Reaction Volume}}{0.0045 \times \text{Enzyme volume} \times \text{Time} \times \text{protein concentration}}$$

$$\text{Total enzyme activity} = \frac{0D420 \times \text{Reaction Volume} \times \text{total protein}}{0.0045 \times \text{Enzyme volume} \times \text{Time} \times \text{protein concentration}}$$

Determination of optimum time duration: 10 test tubes were filled with 750 µl of 0.01M ONPG prepared in phosphate buffer (pH 6.8, 0.1M). To every test tubes 250µl of crude enzyme extract and 2ml of phosphate buffer (pH 6.8, 0.1M) was added to the test tube and then incubated at 37°C for 70 minutes. The reaction was quenched by adding 250µl of 0.5M Na₂CO₃ at every 30 minutes interval and the reaction progress was determined spectrophotometrically at 420nm against

the blank (750 μl ONPG, 2250 μl phosphate buffer of pH 6.8) (Fernandes et al. 2002).

Determination of optimum temperature: 8 test tubes were filled with 750 μl of 0.01M ONPG prepared in phosphate buffer (pH 6.8, 0.1M). To every test tubes 250 μl of crude enzyme extract and 2ml of phosphate buffer (pH 6.8, 0.1M) was added to the test tube and then incubated separately at different temperatures of 0°C, 14°C, 22°C, 27°C, 37°C, 40°C, 45°C and 50°C for 70 minutes. The reaction was quenched by adding 250 μl of 0.5M Na_2CO_3 and the reaction progress was determined spectrophotometrically at 420 nm against the blank (750 μl ONPG, 2250 μl phosphate buffer of pH 6.8) (Zhou and Chen 2001).

Determination of optimum pH: Eight test tubes were filled with 750 μl of 0.01M ONPG prepared in phosphate buffer of pH 5.5, 6.0, 6.2, 6.4, 6.6, 6.8, 7.0 and 7.12. To every test tube 250 μl of crude enzyme extract and 2ml of phosphate buffer of respective pH was added and then incubated at 37°C for 70 minutes. The reaction was quenched by adding 250 μl of 0.5M Na_2CO_3 and the reaction progress was determined spectrophotometrically at 420 nm against the blank (750 μl ONPG, 2250 μl phosphate buffer of pH 6.8) (Zhou and Chen 2001).

Determination of K_m value: Nine test tubes were filled with 50, 100, 150, 200, 250, 300, 350, 400 and 450 μl of 0.01M ONPG prepared in phosphate buffer of pH 6.8. Final volume was made 1750 μl by addition of phosphate buffer of pH 6.8. 250 μl of enzyme was added to each test tubes and incubated at 37°C for 70 min. The reaction was quenched by adding 250 μl of 0.5M Na_2CO_3 and the reaction progress was determined spectrophotometrically at 420 nm against the blank (750 μl ONPG, 1250 μl phosphate buffer of pH 6.8) (Fernandes et al. 2002).

RESULTS

Isolation of lactose positive yeast strains: Four lactose positive yeast isolates were obtained from the dairy samples. Isolate 1 showing the highest lactose positive activity was taken for further study.

Protein concentration: The protein concentration in extracted and purified enzyme extracts was found to be 0.465 $\mu\text{g}/\mu\text{l}$ (mg/ml) using Bradford assay compared with standard BSA solution.

Effect of incubation time, temperature and pH on enzyme activity: The enzyme has an optimum incubation time of 70 min and optimum temperature at 37°C. Whereas the optimum pH of enzyme was found to be slightly acidic at 6.8 (Figure 1, 2 and 3).

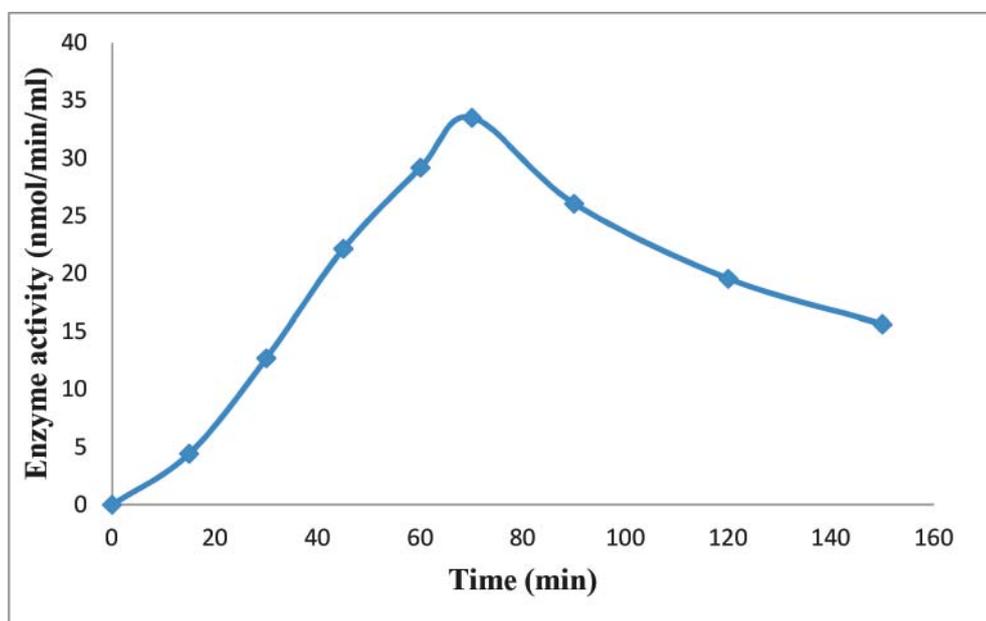


Figure 1: Effect of incubation time on enzyme activity

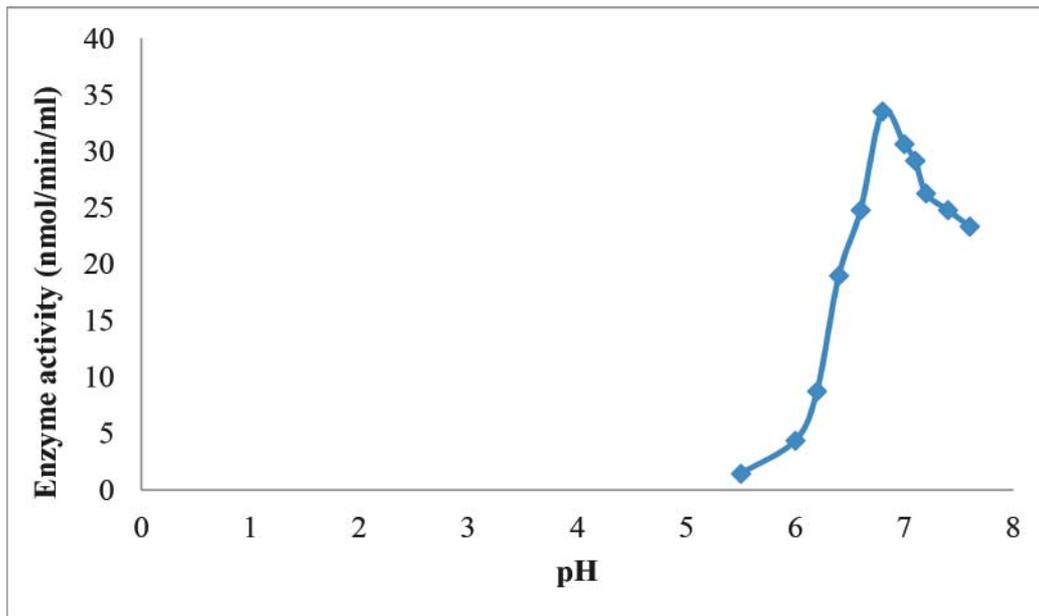


Figure 2: Effect of Temperature on enzyme activity

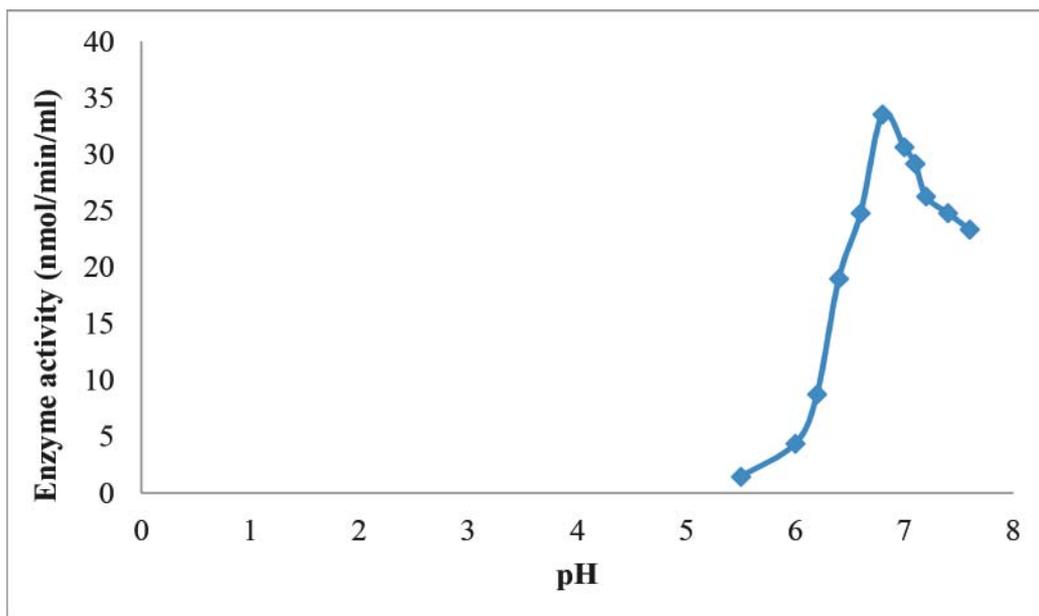


Figure 3: Effect of pH on Enzyme activity

Effect of incubation time, temperature and pH on specific activity: Like an enzyme activity, highest

specific activity of enzyme was observed with 70 min incubation, at 37°C temperature and at 6.8 pH (Table 1).

Table 1: Effect of incubation time, temperature and pH on specific activity of enzyme

Incubation time (min)	Specific activity (nmol/min/mg)	Temperature (°C)	Specific activity (nmol/min/mg)	pH	Specific activity (nmol/min/mg)
0	0.00	0	15.61	5.5	3.10
15	9.53	14	37.63	6.0	9.40
30	27.33	22	53.25	6.2	18.82
45	47.63	27	62.67	6.4	40.73
60	62.75	37	72.06	6.6	53.24
70	72.06	40	37.63	6.8	72.06
90	55.94	45	12.52	7.0	65.84
120	42.01	50	9.40	7.1	62.67
150	33.61			7.2	56.43
				7.4	53.25
				7.6	50.13

Effect of substrate concentration on enzyme activity:

The V_{max} and K_m values of enzymes were determined to 58.82nmol/ min/ml and 2.23mM of ONPG respectively using Lineweaver-Burk plot while V_{max} and K_m values

were found to be 54.43nmol/min/ml and 2.036 mM of ONPG respectively using Eadie-Hofstee plot (Table 2, Figure 4 and Figure 5).

Table 2: Effect of substrate Concentration on enzyme activity

Concentration of substrate [S] (mM)	Enzyme activity (V) (nmol/min/ml)	1/[S]	1/V	V/ [S]
0.285	6.6286	3.5088	0.1509	23.2584
0.571	11.00	1.7513	0.0909	19.2643
0.857	16.7143	1.1669	0.0598	19.5039
1.14	20.6286	0.8772	0.0485	18.0954
1.43	22.0571	0.6993	0.0453	15.4245
1.71	25.5714	0.5848	0.0391	14.9542
2.00	27.2857	0.5000	0.0366	13.6429
2.28	27.8857	0.4386	0.0359	12.2307

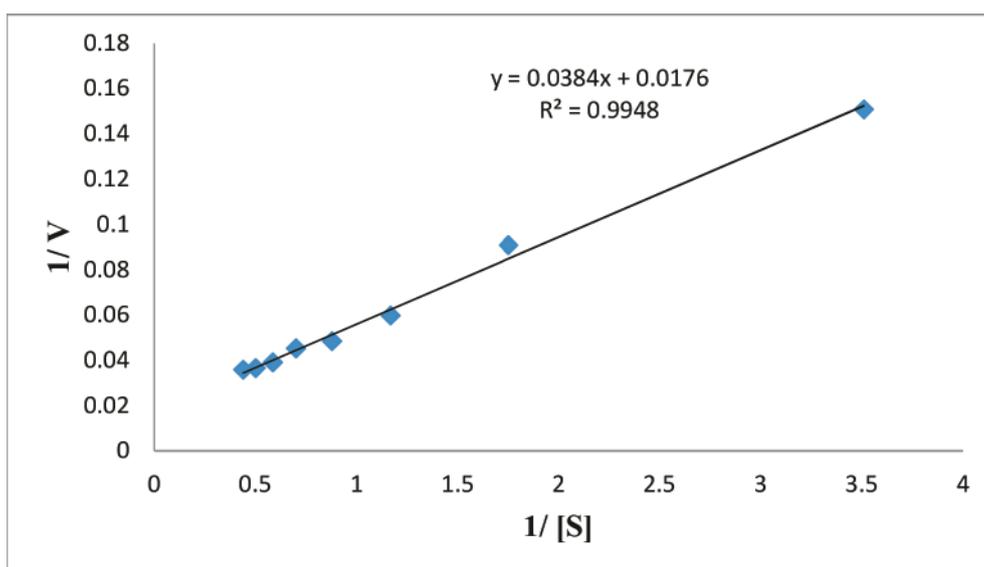


Figure 4: Lineweaver-Burk/Double reciprocal plot

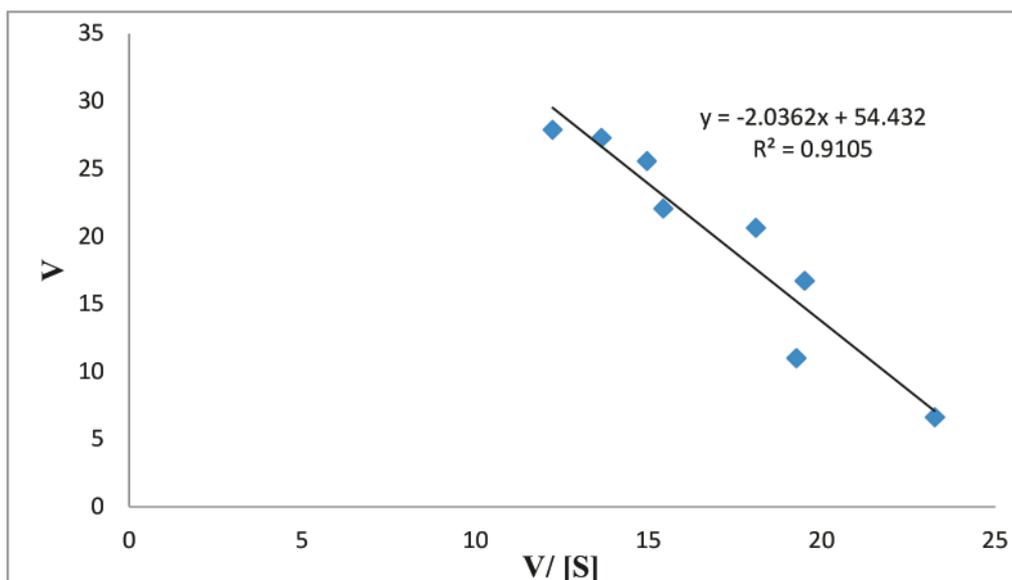


Figure 5: Eadie-Hofstee plot (V/[S] Vs. V)

Calculation of specific and total enzyme activity:
The specific and total enzyme activity were calculated

to be 59.97nmol/min/mg and 3346.33nmol/min respectively (Table 3).

Table 3: Summary of enzyme characteristics

Enzyme parameters	Values
Protein concentration of enzyme extract	0.465 mg/ml
Initial volume of enzyme extract	120 ml
Total protein of enzyme extract	55.8 mg
Optimum time	70 min.
Optimum pH	6.8
Optimum temperature	37°C
K_m	2.23 mM of ONPG
V_{max}	58.82 nmol/min/ml
Enzyme activity	27.88 nmol/min/ml
Specific enzyme activity	59.97 nmol/min/mg
Total enzyme activity	3346.33 nmol/min

DISCUSSION

The knowledge of enzyme stability can provide information on the structure of an enzyme which can aid in the economical industrial production of enzyme. Activity and stability of an enzyme depend upon the spatial conformation of the protein and is affected by various environmental factors such as pH, temperature, reaction medium, presence of ions. It is important to optimize these factors so, as to allow economical industrial production of enzyme. It is also important to determine the enzyme kinetic parameters such as Michaelis-Menten constant (K_m), maximum enzyme activity (V_{max}), optimum time duration which are necessary to characterize an enzyme and to optimize the enzymatic process.

The optimum time of reaction was found to be 70 mins. The activity gradually fell beyond 70 mins indicating the inactivation of the enzyme with time. The activity curve for effect of pH gave a bell-shaped curve with maximum activity in the near neutral pH of 6.8. The optimum pH of 6.8 suggests that the enzyme can be classified as slightly acidic. However, the enzyme showed activity over a wide range of pH from 5.5-7.6. In general, β -galactosidase from yeast and bacteria has optimum pH at near neutral range, whereas the enzyme from molds acts well at more acidic level (Gurr 1987; Sani et al. 1999).

The activity curve of effect of temperature on enzyme activity gave a bell-shaped curve with maximum activity at 37°C. The enzyme had wide range of

working temperature from 0-50°C. However, greater than 50% hydrolyzing ability was maintained in the range of 14-40°C. The enzyme even showed slight activity at freezing temperature (0°C). This shows that the enzyme has potential application for managing the lactose pollution at the normal as well as low ambient temperatures. The enzyme showed least activity at temperatures 45°C or greater. This must be due to the denaturation of the enzyme at higher temperatures (Sani et al. 1999; Wolosowska and Synowiecki 2004).

The activity curve for effect of substrate concentration on enzyme activity (Michaelis-Menten plot) showed that at low substrate concentration, almost linear increment in enzyme activity with increase in substrate concentration was seen. But at higher substrate concentration the enzyme activity increased by smaller and smaller amount in response to increases in substrate concentration and almost a plateau like region was seen. The activity/rate of an enzymatic reaction depends upon the concentration of the enzyme substrate [ES] complex. At low substrate concentration most of the enzyme is present in uncombined form [E], so the rate is proportional to substrate concentration. This explains for almost linear increment in enzyme activity with increase in substrate concentration, at low substrate concentration. At high substrate concentration most of the enzyme is present as ES complex i.e. virtually all the active sites in an enzyme are saturated with substrate, so that further increase in substrate concentration doesn't produce an appreciable increment in the enzyme activity. This explains the almost plateau like region observed in the enzyme activity at higher substrate concentration (Pivarnik et al. 1995; Prenosil et al. 1987).

From Lineweaver-Burk plot the K_m value and V_{max} was found to be 2.23mM of ONPG and 58.82nmol/min/ml respectively. From Eadie-Hofstee plot the K_m value and V_{max} was found to be 2.036 mM of ONPG and 54.43nmol/min/ml respectively. The low K_m value of the enzyme indicated that the enzyme had high affinity for the substrate ONPG and the substrate is tightly bound to the enzyme (Fernandes et al. 2002).

The protein concentration of the crude enzyme extract was found to be 0.465 mg/ml. The low protein concentration and the high enzyme activity indicated that the enzyme β -galactosidase comprised most of the protein fraction of the crude enzyme extract. It might

be due to the induction of β -galactosidase as a result of high lactose content of mass culture broth (YEP broth).

CONCLUSION

The enzyme was slightly acidic (near neutral pH) and showed maximum activity at 37°C but showed activity over a wide range of temperature 0-50°C. K_m value of the enzyme was 2.23 mM of ONPG, thus the enzyme has high affinity for ONPG. The overall properties of enzyme showed the potential use of the enzyme as industrial application.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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