Pakistan Journal of Life and Social Sciences
www.pjlss.edu.pk

https://doi.org/10.57239/PJLSS-2024-22.2.0042

RESEARCH ARTICLE

Characterization of Beta-Galactosidase Enzyme Kinetics and Thermodynamics Produced from Aspergillus Oryzae

Sami Kadhim Hassan *

College of Biotechnology, University of Al-Qadisiyah, IRAQ

ARTICLE INFO

Received: Apr 24, 2024

Accepted: June 13, 2024

Keywords

β-galactosidase enzyme
O-nitrophenyl-β-galactoside substrate
Km
Vmax
PH sensitivity
Thermal stability
Activation energy
Aspergillus oryzae

***Corresponding Author:**

sami.hassan@qu.edu.iq

ABSTRACT

This study investigates the properties of β-galactosidase enzyme derived from Aspergillus oryzae, a mold fungus. The Michaelis-Menten constants (Km and Vmax) were determined using o-nitrophenyl-β-galactoside (ONPG) as the substrate. The Km value, calculated from the Lineweaver-Burk plot, was found to be 0.800 mM, with a Vmax of 0.0864 (A/min). Additional Km and Vmax values were obtained from different plots: Michaelis-Menten (Km = 0.840 mM, Vmax = 0.0838 A/min), Direct Linear (Km = 0.800 mM, Vmax = 0.0850 A/min), Hanes (Km = 0.633 mM, Vmax = 0.1216 A/min), and Eadie-Hofstee (Km = 0.043 mM, Vmax = 0.0867 A/min). The enzymatic activity was explored across a pH range of 5.0 to 8.0, revealing an optimum activity at pH 7.5. The enzyme exhibited a preference for slightly alkaline conditions, as its activity decreased with decreasing pH, reaching zero at pH 4.0. Thermal stability was assessed by determining the activation energy (Ea) and denaturation kinetics. The rate constant of denaturation increased with temperature, ranging from 0.2366 to 0.4768 min^{-1} as the temperature rose from 60 to 62°C. Denaturation times at 60, 61, and 62°C were 20.172, 19.821, and 10.028 min, respectively, indicating enhanced thermal stability at 61 and 61.5°C for 1 and 2 minutes. However, the enzyme showed reduced stability at 62°C after 4 minutes. The activation energy for β-galactosidase inactivation (Ea) was determined as 32,430.63 J/mol.

INTRODUCTION

Beta-galactosidase (EC 3.2.1.23), also known as beta-gal or β-gal, is a hydrolase enzyme that catalyzes the hydrolysis of β-galactosides into monosaccharides. Substrates of β-galactosidases include ganglioside GM1, lactosylceramides, lactose (milk sugar, glucose-galactose), and various glycoproteins. Although often confused with lactase, lactase is actually a sub-class of β-galactosidase.

β-galactosidase is an exoglycosidase that hydrolyzes the β-glycosidic bond between a galactose and its organic moiety. It can also cleave fucosides and arabinosides, but with much lower efficiency. This enzyme is essential in the human body; deficiencies can lead to conditions like galactosialidosis or Morquio B syndrome. In *E. coli*, the gene for β-galactosidase (lacZ) is part of the inducible lac operon system, which is activated in the presence of lactose when glucose levels are low.

The enzyme's active site catalyzes hydrolysis via "shallow" and "deep" binding. Monovalent potassium ions (K^+) and divalent magnesium ions (Mg^{2+}) are required for optimal activity. The beta-linkage of the substrate is cleaved by a terminal carboxyl group on the side chain of a glutamic acid residue.

To facilitate measurements, we use an artificial substrate, o-nitrophenyl-β-galactoside (ONPG), where the chromophore ortho-(or 3-) nitrophenol is linked to beta-galactose by a heteroglycosidic bond. When hydrolyzed, o-nitrophenol is formed. At alkaline pH, the phenolic proton dissociates, forming a phenolate anion with an intense yellow color measurable by spectrophotometry. The o-nitrophenyl group remains non-ionized while covalently bound to galactose; color develops only post-hydrolysis. Such substrates are termed chromogenic (Tanaka et al., 1975).

The β-galactosidase assay is commonly used in genetics, molecular biology, and other life sciences. In the dairy industry, β-galactosidases are used on a large scale, such as in producing low-lactose milk for lactose-intolerant individuals.

The enzyme used in this research is from *Aspergillus oryzae*, a mold fungus. It is primarily used for hydrolyzing lactose in whey.

Enzymes are distinct from other proteins due to their ability to bind specific substrates at their active sites and convert them into products. The rates of catalytic reactions are influenced by several experimental parameters, including substrate concentration, enzyme concentration, pH, temperature, and the presence of inhibitors. The Lineweaver-Burk method is frequently used to determine Km and Vmax, forming a straight line by taking the reciprocal of the Michaelis-Menten equation. When $1/V_0$ is plotted against 1/[S], the y-axis intercept is 1/Vmax, and the x-axis intercept is -1/Km.

However, Eisenthal and Cornish-Bowden (1974) noted that errors in observations could lead to varying Vmax and Km values when different substrate concentrations are used to determine enzyme activity. They suggested the direct linear plot method to address inaccuracies caused by observational errors. This method uses the medians of Vmax and Km values as the best estimates instead of means, reducing the influence of outliers due to poor experimental precision.

The objective of this study was to characterize the activity, kinetics, and thermodynamics of β-galactosidase from *Aspergillus oryzae* in terms of Km and Vmax. The study also aimed to determine the optimum pH and temperature for enzyme activity and to calculate the activation energy (Ea) of thermal inactivation (thermal stability) using o-nitrophenyl-β-galactoside (ONPG) as the substrate.

MATERIALS AND METHODS

Beta-Galactosidase Assay

The β-galactosidase enzyme from *Aspergillus oryzae* was used for the assay. The enzymatic activity was measured spectrophotometrically using a BioSpec-1601 spectrophotometer (Shimadzu Scientific Instruments Inc., Japan) by monitoring the increase in absorbance at 405 nm over 60 seconds at room temperature. The following components were prepared and used in the assay:

- Substrate Buffer: 0.200 mL of 40 mM o-nitrophenyl-β-galactoside (ONPG; MW 301.3 g/mol), 0.120 g dissolved in 10 mL substrate buffer (0.1 M Tris-HCl with 0.01 M magnesium chloride, pH 7.5).

- Enzyme Buffer: Cold enzyme stock was diluted 1/5 with enzyme buffer (0.200 mL of 0.1 mL enzyme β-galactosidase dissolved in 4.9 mL enzyme buffer consisting of 0.1 M Tris-HCl, 0.01 M magnesium chloride, and 0.01 M sodium chloride, pH 7.5).

- Assay Buffer: 0.1 M Tris-HCl containing 0.001 M magnesium chloride, pH 7.5.

Control tubes contained the substrate without the presence of the enzyme. All assays were performed in triplicate (Walsh, 2005; Jam et al., 2010; Tashtoush et al., 2023b).

Generating the Enzyme Kinetic Data

1. Preparation:

- Add 600 µL of assay buffer to a cuvette.

- Add 200 µL of substrate to the cuvette and place it in the spectrophotometer.

- Add 200 µL of enzyme to the same cuvette, pipette up and down, and start the measurement.

2. Blank Preparation:

- Use 800 µL of assay buffer and 200 µL of substrate as the blank.

Data were recorded in a table of results.

Determination of Kinetic Parameters

The Michaelis-Menten constants (Km and Vmax) were determined by measuring the enzymatic activity reaction rates (V, mM/min/mg protein) at various substrate concentrations. Substrate concentrations used were undiluted (39.827 mM) and dilutions of 1/20 (1.991 mM), 1/40 (0.996 mM), 1/50 (0.796 mM), 1/75 (0.531 mM), and 1/100 (0.398 mM) . (Table 2 and Figure 1). Km and Vmax values were calculated using:

- Direct linear plots of the initial rate data (Table 2 and Figure 3)

- Lineweaver-Burk plots (Table 3 and Figure 2)

- Hanes plots (Table 2 and Figure 4)

- Eadie-Hofstee plots (Table 2 and Figure 5)

Linear regression analysis was performed using Microsoft Excel XP to improve the accuracy of the intersection points. Best estimated values of Km and Vmax were determined as the medians of the three sets of estimates at least (Table 1 and Figure 1, 2, 3, 4, 5).

Effect of pH on Enzymatic Activity

Dilute cold enzyme stock 1/5 with enzyme buffer as described in β-galactosidase assay section. The enzyme was assayed in five different assay buffers with pH values of 4, 5, 6, 7.5, and 8. Each buffer contained 0.1 M Tris-HCl and 0.001 M magnesium chloride, adjusted with sodium citrate. The substrate used was 40 mM ONPG with substrate buffer of (0.1 M Tris-HCl contains 0.01 M magnesium chloride, PH 7.5) at room temperature. A new blank with substrate was prepared for each pH tested. Enzyme activity was plotted against pH, and assays were conducted in triplicate. (Walsh, 2005). (Table 4 and Figure 6).

Effect of Thermal Treatment on Enzymatic Activity

Residual enzyme activity was measured after heating the enzyme to 60°C, 61°C, and 62°C for 1, 2, 3, and 4 minutes. The enzyme buffer used was 0.1 M Tris-HCl with 0.01 M magnesium chloride and 0.01 M sodium chloride, pH 7.5. Enzyme samples were cooled to room temperature in an ice bath before measuring residual activity. Use the same 1/5 dilution of the enzyme. Set the water bath to 62°C. The assay was performed in triplicate for each sample using ONPG. Aliquot 0.5 ml of enzyme solution into 4 different microfuge tubes. Place the tubes in the water bath when the temperature reaches 60oC and start the timer. Pull a sample from the water bath each min for 4 min and place in ice. After sample is back to room temperature, remove from ice. After 4 min, assay the activity in at least triplicate of each sample using ONPG (Walsh, 2005; Jam et al., 2011; Tashtoush et al., 2023a).

The residual activity percent was calculated as:

Residual enzyme activity (%) = 100 (Ct/ Co)

Where, Ct is the activity at a certain temperature of time t (min), and Co is the corresponding activity at room temperature (25°C) of the same time.

Set the water bath to 61°C and repeat the above experiment. When done, set the water bath to 60oC and repeat the above experiment.

For assessing the temperature and time for thermal denaturation of the β-galactosidase enzyme, set the water bath to 62°C and pool enzyme samples every minute. The enzyme samples have to be immediately cooled on ice to bring the temperature down to 37°C and then checked for activity. Use average (0.0833) of three values as the room temperature rates for V (A/min): 0.085, 0.087, 0.078. The data should be recorded in Tables 5, 6, 7.

The log (Ln) of the enzyme percent residual activity of the thermally treated enzymes (of the k value) should be graphed vs. the time for each temperature analyzed (Figure 7 drew from Table 7). The slope of these lines is the absolute temperature (Kelvin) k min^{-1} values of denaturation, or the rate constant for denaturation of the enzyme. This data also allows calculation of the time needed at each temperature to completely inactivate the enzyme.

A second graph was produced by plotting the natural log (log_{10}) of the K values at each temperature vs. 1/T (Kelvin) allows calculation energy of the activation energy (Ea) for enzyme inactivation from the slope of the line (Ea/R). Use the R value of 8.3144 J/mol K. This is described in Whitaker, J.R. 1996. (Figure 8 drew from X-axis and Y axis data of Table 8).

RESULTS AND DISCUSSION

Kinetic properties of β-galactosidase

The activity of β-galactosidase was measured across a series of substrate concentrations using o-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate. The Michaelis-Menten constant (Km) and the maximal velocity (Vmax) of the reaction were estimated using various graphical methods: Michaelis-Menten, Lineweaver-Burk, Direct Linear, Hanes, and Eadie-Hofstee plots. The values of km is 0.840, 0.800, 0.800, 0.633, and 0.043 mM respectively, while the V max activity 0.0838, 0.0864, 0.0850, 0.1216, and 0.0867 (A/min) respectively (Table 1, Table 2, and Figure 1, 2, 3, 4, 5) by using o-nitrophneyl-b-galactoside (ONPG) as substrate. The use of Lineweaver–Burk plot ignores the error which the observation can be subject to. In this assumption, all the experimental lines intersect at a common point, whose coordinates (Km, V max) provide the values satisfying the Michaelis–Menten equation exactly for each observation (Eisenthal and Cornish-Bowden, 1974; Jarrah et al., 2022b; Zakariya et al., 2023; Alneyadi et al., 2023b).

The Michaelis-Menten plot (Figure 1) showed a Km value of approximately 0.840 mM and a Vmax of 0.0838 mM/min/mg protein (A/min), which is lower than the Vmax of 0.345 mM/min/mg reported by Greenberg and Mahoney (1981). The Lineweaver-Burk plot (Figure 2) yielded a Km of 0.800 mM and a Vmax of 0.0864 A/min (Table 1 and Figure 2).These Km values are comparable to the 0.56 mM reported for β-galactosidase from Aspergillus carbonarius by O'Connell and Walsh (2008) but higher than the 0.30 mM found by Wallenfels and Weil (1972), and less than b- galactosidase from Aspergillus niger for each of Greenberg and Mahoney (1981) (2.02 mM), and O'Connell and Walsh (2008) (1.74 mM), and of Martarello et, al., (2019) (1.84 mM). Conversely, they are significantly lower than those reported for β-galactosidase from Aspergillus niger and E. coli using ONPG as the substrate.

As suggested by Eisenthal and Cornish-Bowden (1974), the use of the double reciprocal plot (Lineweaver-Burk) can lead to less accurate estimates unless experimental data are highly precise.

Direct linear and modified direct plot methods offer better reliability by minimizing the influence of outliers.

Effect of pH on β-Galactosidase Activity

The effect of pH on β-galactosidase activity was studied within the pH range of 4.0 to 8.0. The activity profile showed a bell-shaped curve, with maximum activity observed at pH 7.5 (0.0956 A/min) (Table 4 and Figure 6). Activity decreased significantly at pH 5.0 (0.0025 A/min) and reduced to zero at pH 4 suggesting a strong sensitivity to acidic conditions, while at pH 6 and pH 8 the activity reduced to 0.0542 and 0.0884 A/min respectively. This indicates a preference for a slightly alkaline environment, consistent with previous studies showing optimal activity around pH 7.0-7.5 (Table 4 and Figure 6).

Previous study suggested that the optimum pH 7.0-7.5, while the pH stability at pH 6.5- 8.5 (25 C, 20 hr) (Wallenfels and Weil, 1972; Wardat et al., 2024; Alneyadi et al., 2023a). While other found an optimal pH of 7.7 for β- galactosidase activity.
The properties of a number of commercial microbial β-galactosidases are summarized in Table 10. From these data it can be concluded that the optimum pH is between 3.0- 6.5.

Thermal Inactivation of β-Galactosidase

Rat constant of denaturation of enzyme
The rate constant of denaturation of the enzyme was determined by plotting the natural log of the percent activity left (residual) of the thermally treated enzyme against the time for each temperature analyzed. The activity was measured at every 1 min for 4 min. The rate of loss of activity was considered to be first order since pure enzyme was used (Whitaker, 1996). Occasionally, the thermally treatment could introduce a sudden increase or decrease of the activity at a certain interval.

The inclusion of such data points often resulted in an equation with very low correlation coefficient value for the rate of loss of activity. Therefore, such data points were not used in the determination of the equation of loss of activity. As shown in Table 5, 6, 7 and Figure 7, the rate of denaturation of β-galactosidase varies at different temperatures. At 60^0C, after 1 min incubation the enzymatic activity did not have very dramatic change. The mean of residual activity was 97.2 %, while the activity at 62^{0C} after 1 minute the reduction of enzymatic activity became more visible and the mean of residual activity 76.8 %. The enzyme activity decreased more with increase of the time and temperature of incubation. When the β-galactosidase enzyme was incubated at 62^0C, the reduction of activity increased rapidly and the enzyme mean of percent activity left activity 28.4 % and 18.4 % after 3 and 4 minute of incubation respectively (Table 8). Denaturation of protein due to the increase of temperature is usually irreversible.

An estimation of the rate constant of Kelvin (K) for denaturation of the enzyme could be found as the slope of the three lines at 60, 61 and 62^0C (Figure 7). As the temperature increased from 60, 61 and 62^0C, the k value increased from 0.2366, 0.2348 and 0.4768 min^{-1} (Table 8 and 9). Meanwhile, the time needed at each temperature to completely inactivate the enzyme could also calculate from the graph. About 20.172, 19.821, 10.028 min were needed for total denaturation at the temperature of 60, 61 and 62^0C respectively (Table 8), β-galactosidase became thermally instable. Within 4 min of incubation at 62^0C, the enzyme could loss 81.6% of its activity (Table 6).

Activation energy for enzyme inactivation

Fig. 8 shows the plot describing the heat-incativation of β-galactosidase at 60, 61 and 62 ^{0}C, according to the Arrhenius equation: $\ln K = -(Ea/R)(1/T)+C$, where, k is the rate constant of denaturation of

the β-galactosidase enzyme; Ea is the activation energy for enzyme inactivation; T is the corresponding temperature expressed in Kelvin; R value equals 8.3144 J/mol K. The heat inactivation of β-galactosidase was linear. The activation energy (Ea) value was calculated from the slope of the best fit line (Y = 50.25 – 16960.49X, R^2 = 0.7430).

The activation energy for enzyme inactivation was found to be 32,430.63 J/mol (Figure 8). This Ea value was near to 35,700 J/mol for the same enzyme produced from Fusarium moniliform grown in whey found with same ONPG enzyme substrate by Macris and Markakis (1981)[9] and it is inside the typical Ea value for enzyme-catalyzed reactions, which range from 25,000 to 50,000 J/mol (Whitaker, 1996).

CONCLUSION

In conclusion, the study comprehensively investigated the β-galactosidase activity using o-nitrophenyl-β-galactoside (ONPG) as a substrate. Key findings include the determination of the Km value at 0.800 mM and the Vmax at 0.0864 A/min through Lineweaver-Burk analysis. These kinetic parameters were compared with values from previous studies, highlighting variations in Km values, which suggest potential differences in experimental conditions or enzyme sources.

The enzymatic activity was assessed over a pH range of 5.0 to 8.0, revealing a preference for slightly alkaline conditions. The highest activity was recorded at pH 7.5 (0.0956 A/min), with a notable decrease at pH 5.0 (0.0025 A/min) and complete inactivation at pH 4.0, indicating significant sensitivity to acidic environments.

Thermal stability assessments showed that β-galactosidase maintained relative stability at temperatures of 61°C and 62°C for up to 2 minutes, but stability decreased significantly at 62°C after 4 minutes. The rate constant of denaturation and the denaturation time provided further insights into the enzyme's thermal stability.

Additionally, the activation energy for β-galactosidase inactivation (Ea) was calculated to be 32,430.63 J/mol. These findings enhance our understanding of the kinetic and thermodynamic properties of β-galactosidase, offering valuable information for its potential industrial applications and setting a foundation for future enzymology research.

References

Alneyadi, S., Abulibdeh, E., & Wardat, Y. (2023b). The impact of digital environment vs. traditional method on literacy skills; reading and writing of Emirati fourth graders. Sustainability, 15(4), 3418. https://doi.org/10.3390/su15043418

Alneyadi, S., Wardat, Y., Alshannag, Q., & Abu-Al-Aish, A. (2023a). The effect of using smart e-learning app on the academic achievement of eighth-grade students. Eurasia Journal of Mathematics, Science and Technology Education, 19(4), em2248. https://doi.org/10.29333/ejmste/13067

Beta-galactosidase-wikipedia, the free encyclopedia. Characterization of Beta-galactosidase. http://en.wikipedia.org/wiki/Beta-galactosidase.

B-galactosidase km and V max. PDF. Results the hydrolytic cleavage-of o-nitrophenyl galactoside. *www.life.illinois.edu/biochem/355/quizkeys/**b**-gal_lab.pdf*

B-galactosidase review questions. http://biology.kenyon.edu/cources/biolog/betagal/question.htm

Borglum, G. B., and M. Z. Sternberg. 1972. Properties of a fungal lactase. J. Food Sci. 37:619-623.

Cardoso BB, Silvério SC, Abrunhosa L, Teixeira JA, Rodrigues LR (2017) β- galactosidase from Aspergillus lacticoffeatus: a promising biocatalyst for the synthesis of novel prebiotics. Int J Food Microbiol 257:67–74.

Eisenthal, R.and Cornish-Bowden, A. (1974). The Direct Linear Plot. Biochem J. 139:715-720.

Eustice DC, Feldman PA, Colberg-Poley AM, Buckery RM, Neubauer RH. (1991). A sensitive method for the detection of beta-glactosidase in treansfected mammalian cells. Biotechniques. 11 (6): 739-40, 742-3.

Greenberg N. A, Mahoney R. R (1981). Rapid purification of β galactosidase (Asergillus Niger) from a commercial preparation. Journal of Food Science 46 (3):684-687.

Jam, F. A., Akhtar, S., Haq, I. U., Ahmad-U-Rehman, M., & Hijazi, S. T. (2010). Impact of leader behavior on employee job stress: evidence from Pakistan. European Journal of Economics, Finance and Administrative Sciences, (21), 172-179.

Jam, F.A., Khan, T.I., Zaidi, B., & Muzaffar, S.M. (2011). Political Skills Moderates the Relationship between Perception of Organizational Politics and Job Outcomes.

Jarrah, A. M., Wardat, Y., & Gningue, S. (2022b). Misconception on addition and subtraction of fractions in seventh-grade middle school students. Eurasia Journal of Mathematics, Science and Technology Education, 18(6), em2115. https://doi.org/10.29333/ejmste/12070

Macris J. B. and Markakis P. 1981. Characterization of extracellular b-D- galactosidase from fusarium moniliforme grown in whey. J. Applied and Environmental Microbiology, Vol. 41, No. 4. P.956-958.

Martarello R. DA, Martarello R. DA, Cunha L, Cardoso S,L, de Freitas M. M, Damaris Silveira, Fonseca Bazzo Y. M, de Mello M. H, Filho,E. X. F, and Magalhães P. O (2019). Optimization and partial purification of beta-galactosidase production by Aspergillus niger isolated from Brazilian soils using soybean residue. AMB Express 9:81. https://doi.org/10.1186/s13568-019-0805-6

Niu D, Tian X, Mchunu NP, Jia C, Singh S, Liu X, Prior BA, Lu F (2017) Biochemi cal characterization of three Aspergillus niger β-galactosidases. Electron J Biotechnol 27:37–43. https://doi.org/10.1016/j.ejbt.2017.03.001

O'Connell S, Walsh G (2008) Application relevant studies of fungal β galactosidases with potential application in the alleviation of lactose intolerance. Appl Biochem Biotechnol 149(2):129–138. https://doi. org/10.1007/s12010 007 8098-7

O'Connell S, Walsh G (2010) A novel acid stable, acid active β galactosidase potentially suited to the alleviation of lactose intolerance. Appl Microbiol Biotechnol 86 (2):517–524. https://doi.org/10.1007/s00253 009 2270-7

Pastore, G. M., and Y. K. Park. 1979. Screening of high. β--galactosidase-producing fungi and characterizing the hydrolysis properties of a selected strain. J. Food Sci. 44:1577-1580.

Tanaka, Y., Kagamaiishi, A., Kiuchi, A. and Horiuchi, T. (1975) Purification and properties of beta-galactosidase from Aspergillus oryzae .J. Biochem. (Tokyo) 77, 241, 247.

Tashtoush, M. A., AlAli, R., Wardat, Y., Alshraifin, N., & Toubat, H. (2023b). The impact of information and communication technologies (ICT)-based education on the mathematics academic enthusiasm. Journal of Educational and Social Research, 13(3), 284. https://doi.org/10.36941/jesr-2023-0077

Tashtoush, M. A., Wardat, Y., & Elsayed, A. M. (2023a). Mathematics distance learning and learning loss during COVID-19 pandemic: Teachers' perspectives. Journal of Higher Education Theory and Practice, 23(5). https://doi.org/10.33423/jhetp.v23i5.5933

Wallenfels, K. and Weil, R.; The enzymes, Vol.7, P 617 (P.D. Boyer ed), Academic press, New York-London (1972). http://www4.mpbio.com/ecom/docs/proddata.nsf/(webtds2)/104939

Wallerstein Co. 1974. Lactase LP. Technical bulletin no. 406.

Walsh, MK. (2005). Unpublished data. Characterization of Carbonic Anhydrase B activity. Department of Nutrition and food science. Utah State University. Logan, UT.

Wardat, Y., Tashtoush, M., AlAli, R., & Saleh, S. (2024). Artificial intelligence in education: Mathematics teachers' perspectives, practices and challenges. Iraqi Journal for Computer Science and Mathematics, 5(1), 60-77.

Whitaker, R. (1996). Enzyme. Ln: Fennema OR, editor. Food chemistry. 3rd ed. New York: Marcel Dekker, Inc. p 431-530.

Wierzbicki, L. E., and F. V. Kosikowski. 1973. Kinetic of lactose hydrolysis in acid whey by, B-galactosidase from Aspergillus niger. J. Dairy Sci. 56:1396-1399.

Wierzbicki, L. E., and F. V. Kosikowski. 1973. Lactase potential of various microorganisms grown in whey. J. Dairy Sci. 56:26-32.

Zakariya, Y. F., & Wardat, Y. (2023). Job satisfaction of mathematics teachers: An empirical investigation to quantify the contributions of teacher self-efficacy and teacher motivation to teach. Mathematics Education Research Journal. https://doi.org/10.1007/s13394-023-00475-9

APPENDIX

Table 1. Michaelis constant (Km) and the maximal velocity (V max) of the catalytic reaction with β-galactosidase using o-nitophenyl-b-galactoside as substrate.

Method	Km (mM)	Vmax (A/min)
Michaelis-Menten plot	0.840	0.0838
Lineweaver-Burk plot	0.800	0.0864
Direct linear plot	0.800	0.0850
Hanes plot	0.633	0.1216
Eadie-Hofstee plot	0.043	0.0867

Table 2. Enzyme activity vs. o-nitrophenyle-b-galactoside dilution.

Concentration of ONPG [S] (mM)	(V) per mM/min/mg = Activity (A/min)				Mean (A/min)
	Rep 1	Rep 2	Rep 3	Rep 4	
39.827 No dilution	0.0872	0.0819	0.0824	0.0835	0.0838
1.991= 1/20 dilution	0.0659	0.0692	0.0586	0.0607	0.0636
0.996= 1/40 dilution	0.0493	0.0453	0.047	0.0501	0.0479
0.796= 1/50 dilution	0.045	0.0441	0.0406	0.0415	0.0428
0.531=1/75 dilution	0.0373	0.032	0.0319	0.033	0.0335
0.398= 1/100 dilution	0.027	0.0285	0.029	0.028	0.0281

Table 3. Enzyme activity vs. o-nitrophenyle-b-galactoside dilution

1/[S] = 1/ Concentration of ONPG (mM^{-1})	$1/v = (1/Activity)\ (A/min)^{-1}$				
	Rep 1	Rep 2	Rep 3	Rep 4	Mean
0.0251	11.467	12.21	12.136	11.976	11.947
0.5021	15.175	14.451	17.065	16.474	15.791
1.0043	20.284	22.075	21.277	19.96	20.899
1.255	22.222	22.676	24.631	24.096	23.406
1.883	26.81	31.25	31.348	30.303	29.927
2.51	37.037	35.088	34.483	35.714	35.58

Table 4. Enzyme activity measured incubation at different pH.

	Activity (A/min)				
pH	Rep 1	Rep 2	Rep 3	Rep 4	Mean
4	0	0	0	0	0
5	0.003	0.0005	0.0054	0.0012	0.0025
6	0.0536	0.0519	0.0581	0.053	0.0542
7.5	0.0939	0.0951	0.095	0.0985	0.0956
8	0.0905	0.0886	0.088	0.0865	0.0884

Table 5. Enzyme activity measured after incubation at different temperatures.

Incubation temperature ^{o}C	Percent activity left at different incubation time (A/min)			
	1	2	3	4
60 (1)	0.079	0.058	0.045	0.04
60 (2)	0.078	0.06	0.047	0.042
60 (3)	0.086	0.063	0.042	0.04
61 (1)	0.07	0.052	0.048	0.025
61 (2)	0.071	0.05	0.047	0.04
61 (3)	0.068	0.053	0.048	0.035
62 (1)	0.065	0.038	0.02	0.016
62 (2)	0.066	0.033	0.026	0.018
62 (3)	0.061	0.039	0.025	0.012

Table 6: Percent activity left after incubation at different temperatures. (Residual enzyme activity (%)= 100(Ct/C$_0$).

Where, C_t is the activity at a certain temperature of time t (min), and C_o is the corresponding activity at room temperature of the same time.

Incubation temperature ^{o}C	Percent activity left at different incubation times (A/min)				Mean of percent activity loss at 4 min incubation time (A/min)
	1	2	3	4	
60 (1)	94.8	69.6	54	48	
60 (2)	93.6	72	56.4	50.4	
60 (3)	103.2	75.6	50.4	48	
Mean	97.20	62.4	53.6	48.8	100 - 48.8 = 51.2
61 (1)	84	62.4	57.6	30	
61 (2)	85.2	60	56.4	48	
61 (3)	81.6	63.6	57.6	42	
Mean	83.6	62	57.2	40	100 - 40 = 60
62 (1)	78	45.6	24	19.2	
62 (2)	79.2	39.6	31.2	21.6	
62 (3)	73.2	46.8	30	14.4	
Mean	76.8	44	28.4	18.4	100 - 18.4 = 81.6

Note: Table 7 data got from Table 6 by using value 0.0833 A/min of C_0 = V (A/min) as average for three reading at room temp rates (0.085, 0.087, 0.078) and Ct= V (A/min) at 60,or 61 or 62^{o}C. e.g. Ct at 60^{o}C (1) for 1 min = 0.079 (A/min), so Residual enzyme activity(%) =100(0.079/0.0833)= 94.8 A/min.

Table 7. Log (Ln) of the percent activity left after incubation at different temperatures.

Incubation temperature ^{o}C	Ln of Percent activity left at different incubation times (A/min)			
	1	2	3	4
60 (1)	4.5517	4.2427	3.9889	3.8712
60 (2)	4.5390	4.2766	4.0324	3.9199
60 (3)	4.6366	4.3254	3.9199	3.8712

61 (1)	4.4308	4.1335	4.0535	3.4011
61 (2)	4.4450	4.0943	4.0324	3.8712
61 (3)	4.4018	4.1526	4.0535	3.7376
62 (1)	4.3567	3.8199	3.1780	2.9549
62 (2)	4.3719	3.6788	3.4404	3.0726
62 (3)	4.2931	3.8458	3.4011	2.6672

Note: Table 7 data got from data of Table 6 by using Ln of values

Table 8. Estimated of the time to inactivate enzyme.

Equation of Ln (% activity left) at each temperature			Time to inactive enzyme (min) (For total denaturation)	K of denaturation (min^{-1})
T(^oC)	R^2	Line		
60	0.9391	Y = 4.7728 – 0.2366X	4.7728/0.23660 = 20.172	0.2366
61	0.8334	Y = 4.6540 – 0.2348X	4.6540/0.23480 = 19.821	0.2348
62	0.9554	Y = 4.7822 – 0.47687X	4.7822/0.47687 = 10.028	0.4768

Note: Table 8 data got from Figure 7.

Table 9. Effect of temperature on rate constant of the reaction of β-galactosidase with o-nitrophenyl b-galactoside as substrate. Plotted as natural log K (log_{10} K versus 1/T = (1/K) to permit determination of Ea.

Temp. (^0C)	Temp. per K (min^{-1}) from K=(^0C x373/100)+273	1/K (X-axis)	lines Y in Fig. 7 contain K values of denaturation	K of denaturation (min^{-1})	Natural Log of K (Log_{10} K) (Y-axis)
60	496.8=60x373/100+273	0.002012882	Y=4.7728-0.2366X	0.2366	-0.62598
61	500.53=61x373/100+273	0.001997882	Y=4.654-0.2348X	0.2348	-0.62930
62	504.26=62x373/100+273	0.001983104	Y=4.7822-0.4768X	0.4768	-0.32166

Note: Table 9 data got from some data in Table 6, Figure 7 and other calculations.

Table 10. Comparision of Aspergillus oryzae extracellular lactase with certain commercial β–galatosidases

Microorganism	Growth medium	Enzyme location	Optimum		Reference No.
			pH	Temp (^0C)	
Aspergillus foetidus	Not reported	Extracellular	3.5-4.0	66-67	4 -1972
Saccharomyces fragilis	Whey	Cell bound	6.3-6.5	35-45	20 -1973
Aspergillus niger	Wheat bran	Not reported	3.5-4.5	55	21 -1073
Scopulariopsis sp.	Wheat bran	Extracellular	3.6-5.0	50-65	14 -1979
Fusarium moniliforme	Whey	Extraceliular	3.8-5.0	50-60	9 -1981Macris
Aspergillus carbanarius	Not reported	Not reported	3.0-5.0	55-65	13 -2008
Aspergillus Lacticoffeatus	Not reported	Not reported	3.5-4.5	50-60	5 -2017

Aspergillus niger	Not reported	Not reported	4-5	50	11 -2017
Aspergillus niger	Soybean reidue	Extracellular	5.0	50	10 -2019
Aspergillus oryzae	(Wheat bran)	Extraceliular	7.5	50-55	2024 Me

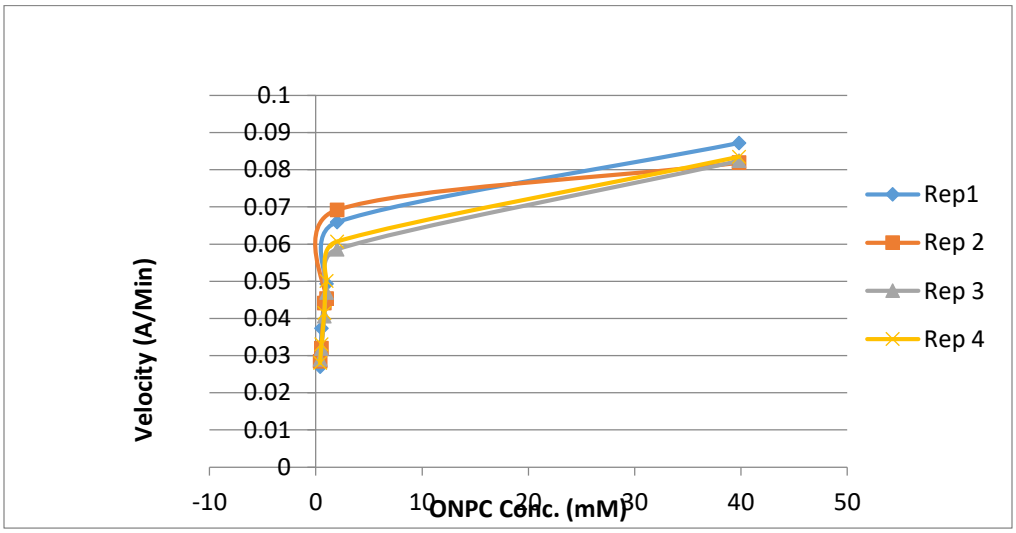

Figure 1. Michaelis–Menten plot for data β–galactosidase with o–nitrophenyl -b–galactoside substrate at different concentration

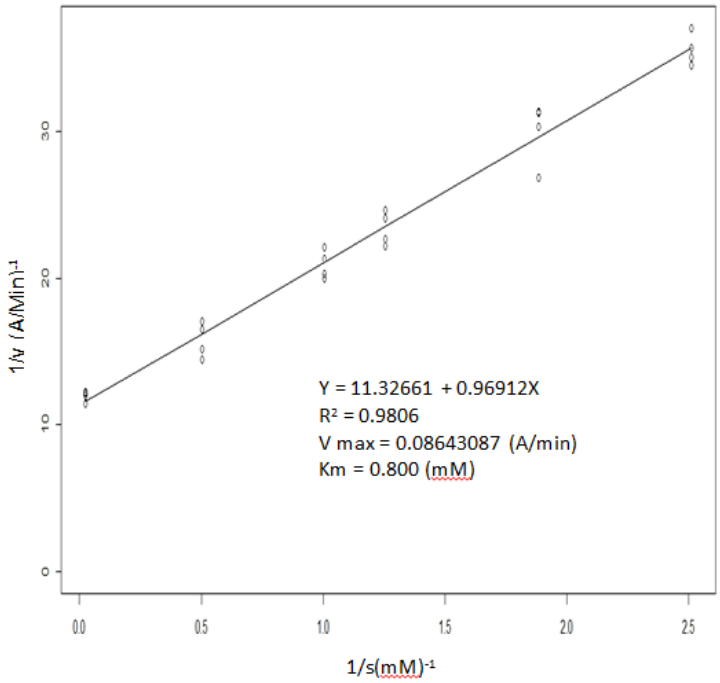

Figure 2. Lineweaver-Burk Plot for β-galactosidase with o–nitrophneyl-b-galactoside substrate at different concentration.

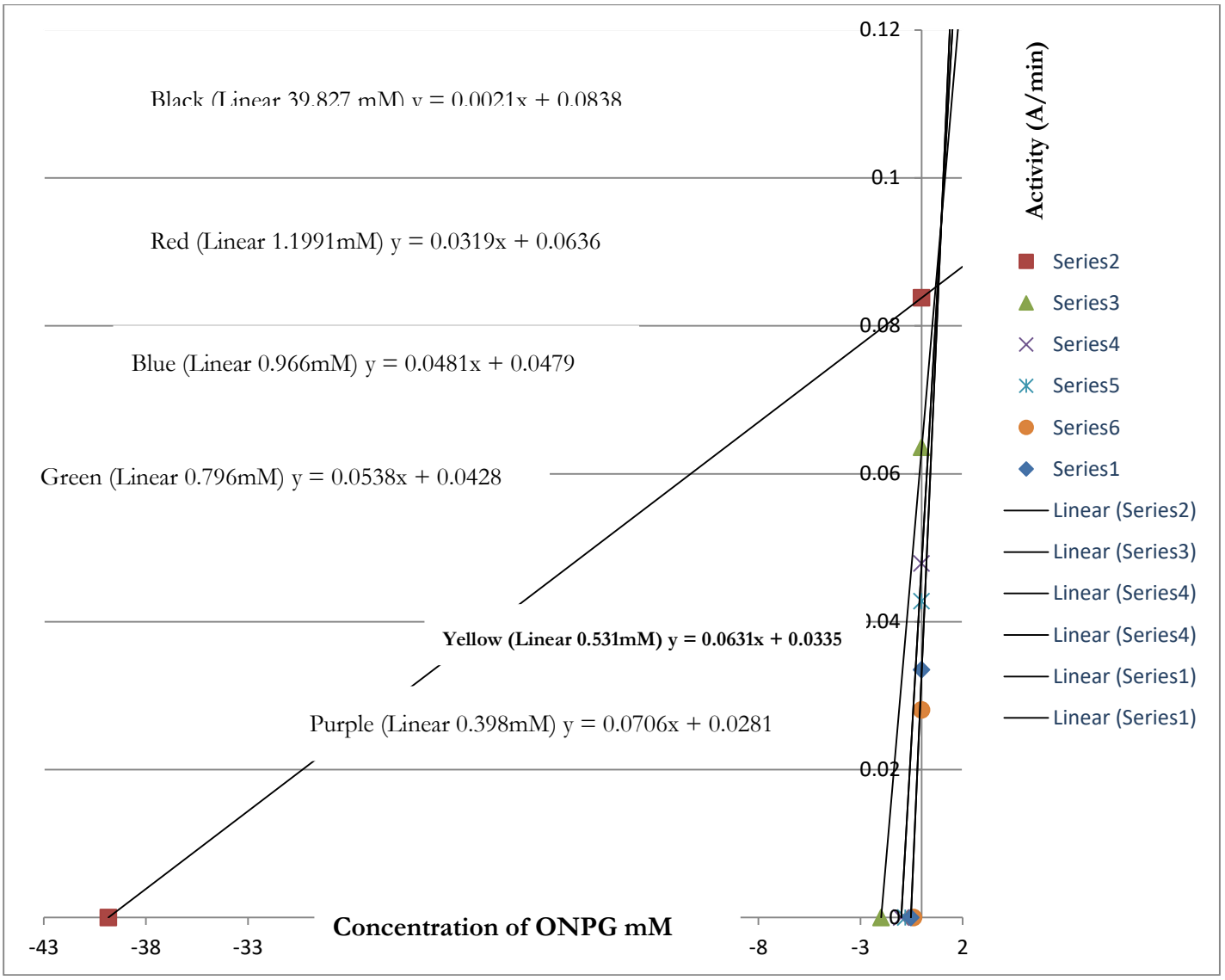

Figure 3. Direct linear plot for β -galatosidase activity with o-nitrophenyl-b-galactoside substrate at different concentration.

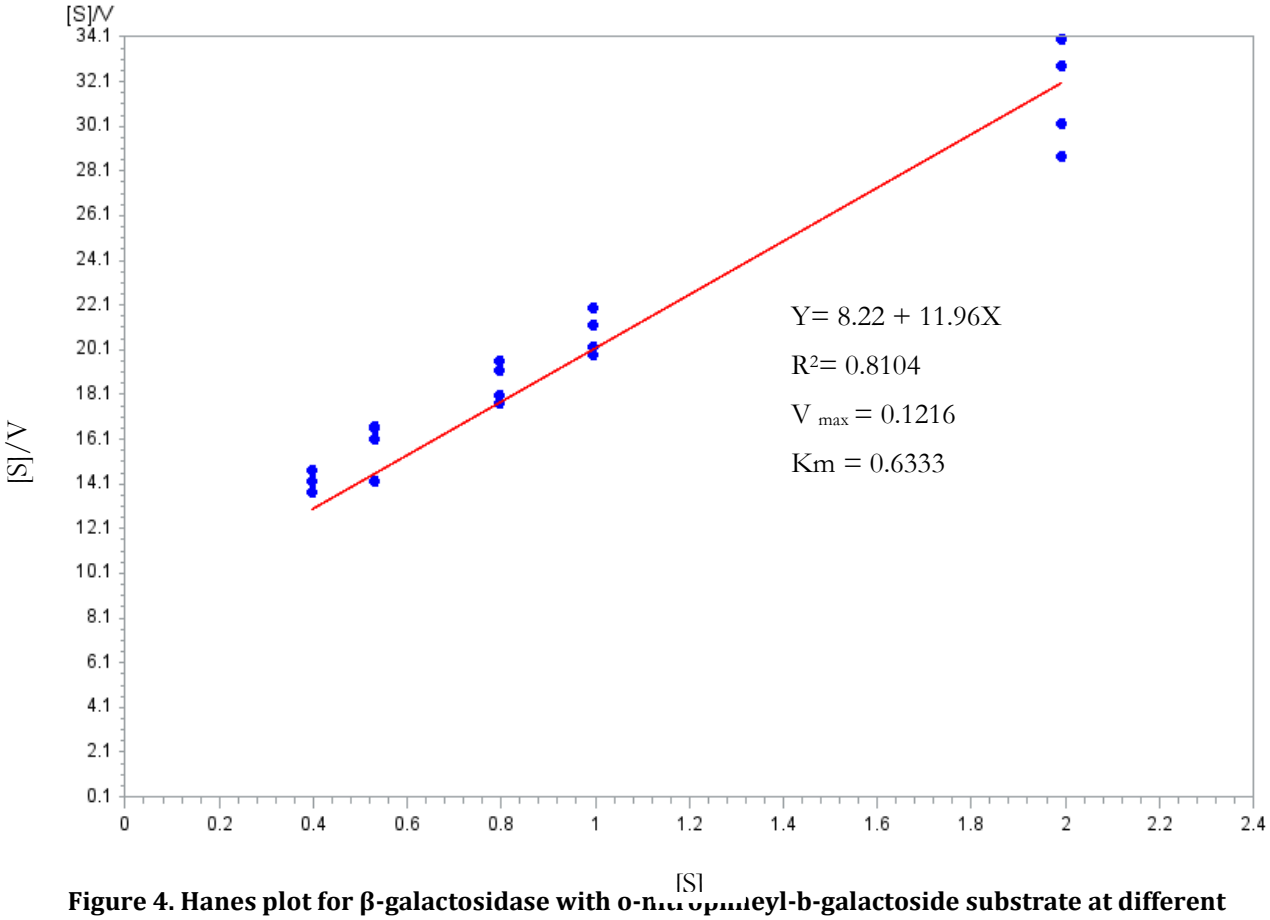

Figure 4. Hanes plot for β-galactosidase with o-nitrophenyl-b-galactoside substrate at different concentration.

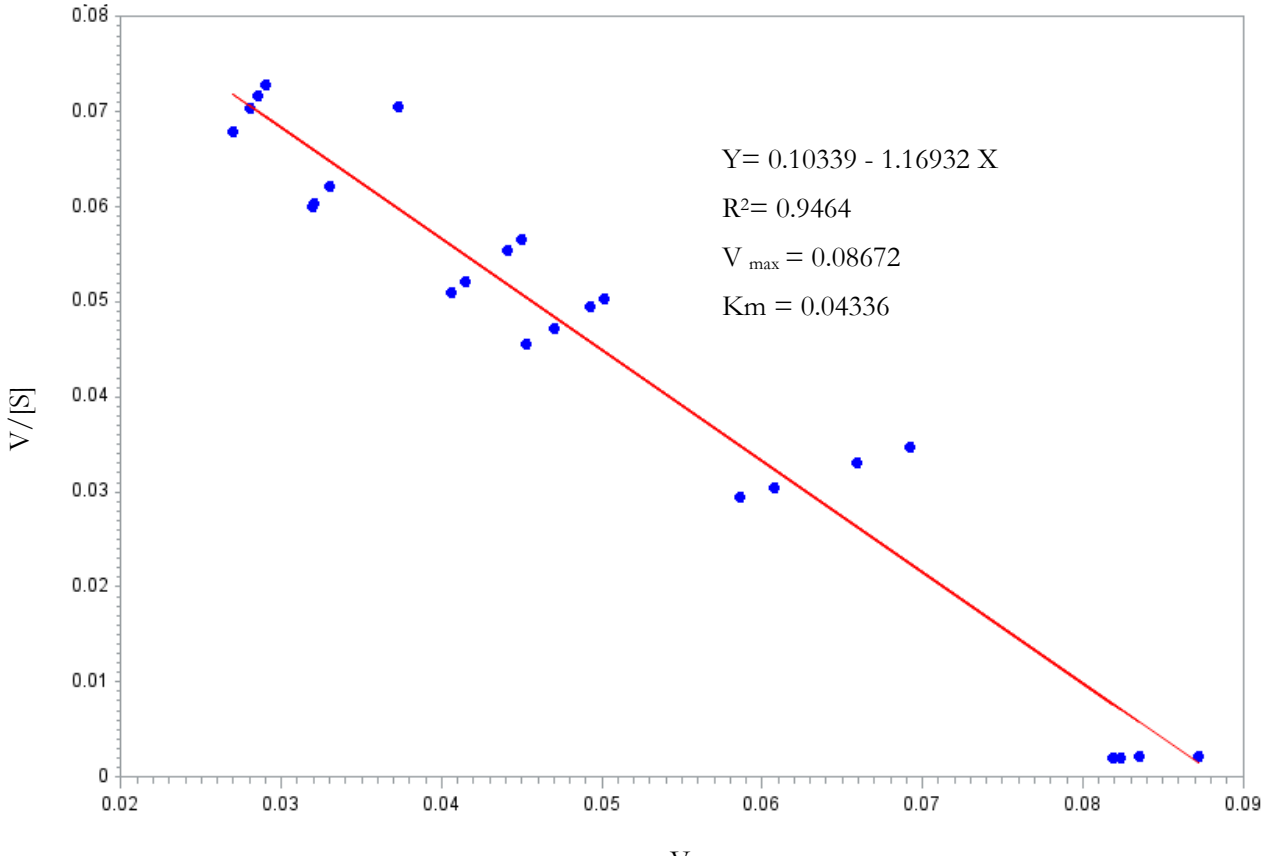

Figure 5. Eadie-Hofstee plot for β-galactosidase with o-nitrophneyl-b-galactoside substrate at different concentration.

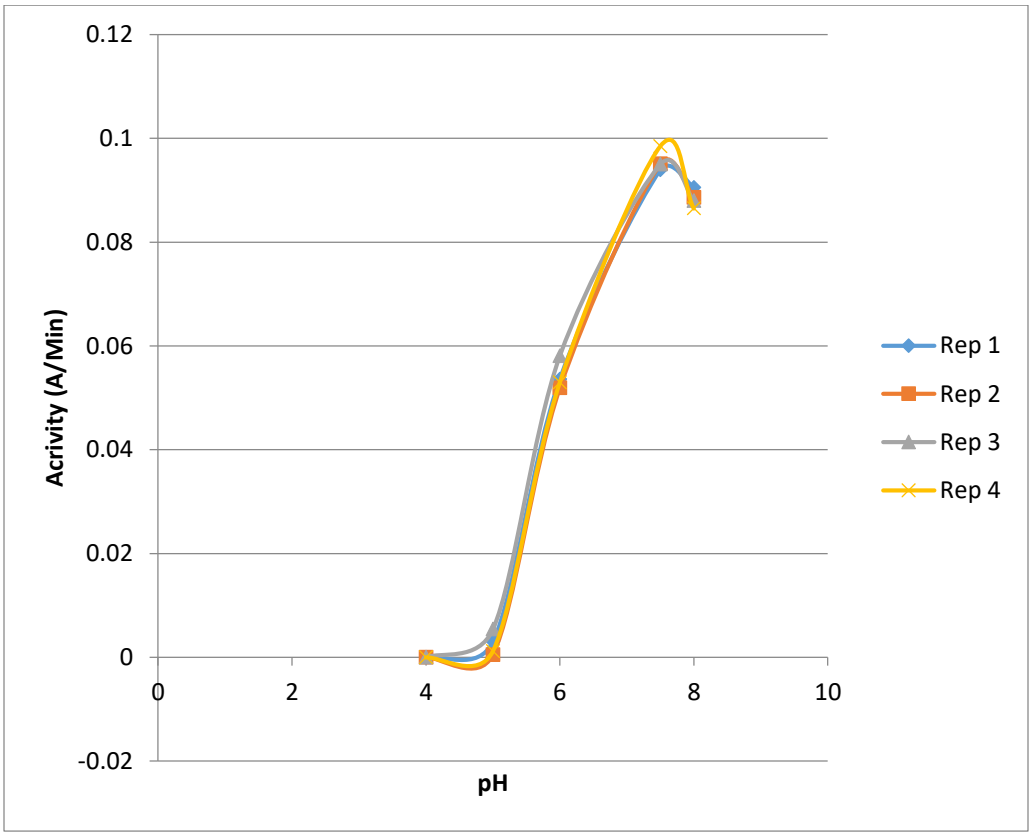

Figure 6. Effect of pH on the β-galactosidase activity with o-nietrophenyl-b-galactoside as substrate.
Figure 6 drew from data of Table 4,

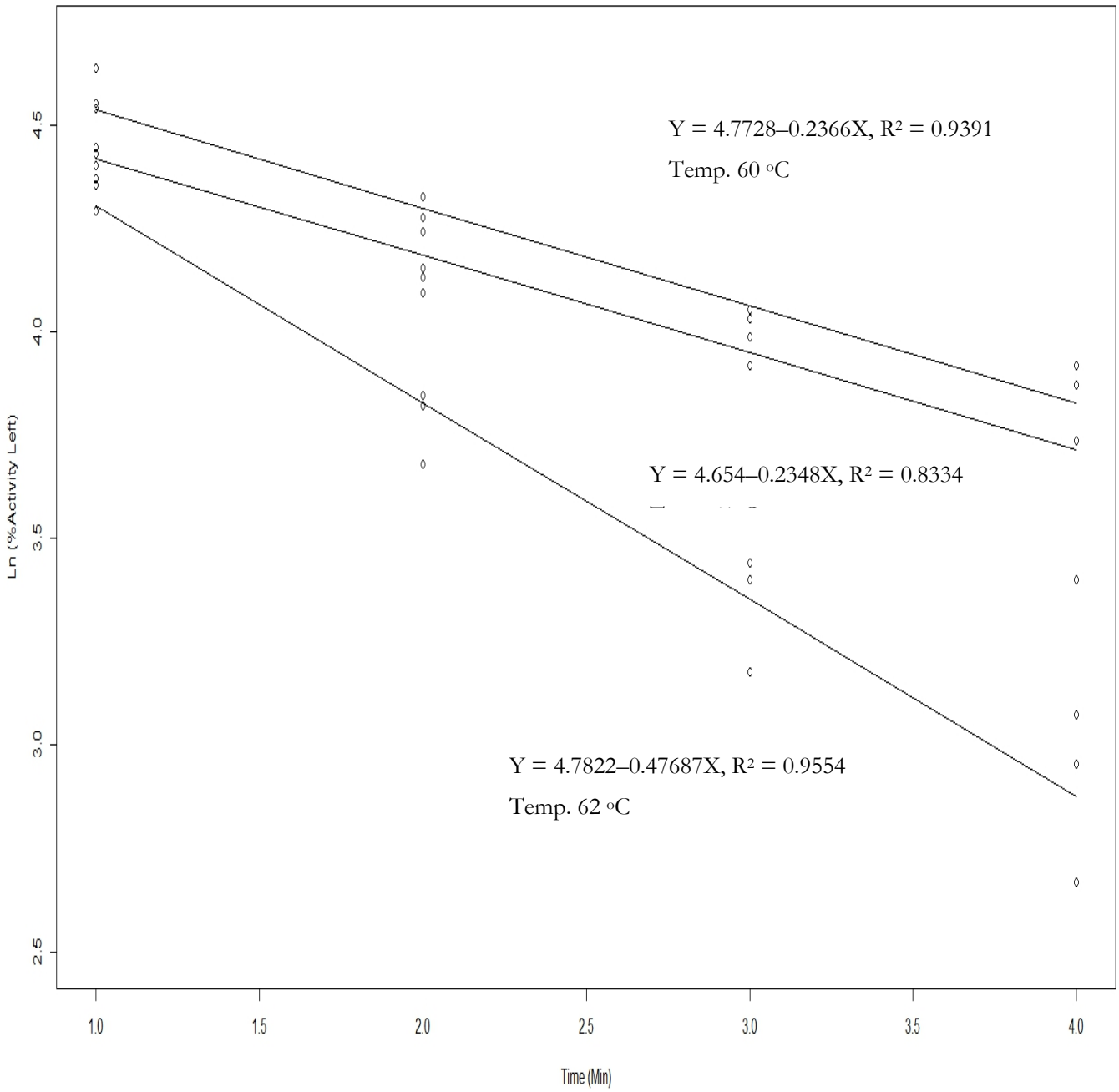

Figure 7 . Rate of denaturation of β-galactosidase at various temperature. Plotted as ln (% activity left) versus time (min). (Figure 7 drew from data of Table 7).

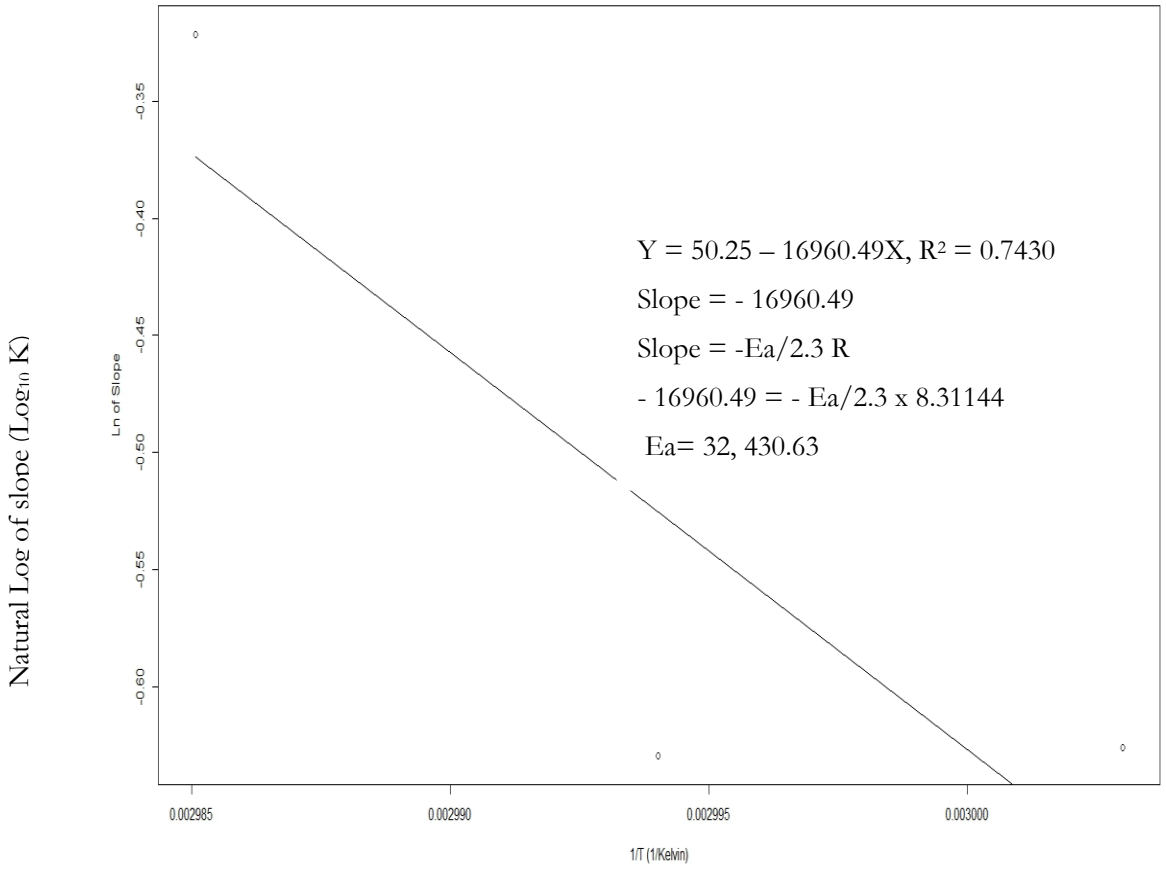

Figure 8. Effect of temperature on rate constant of the reaction of β-galactosidase with o-nitrophenyl b-galactoside as substrate. Plotted as natural log K (log_{10} K versus 1/T = (1/K) to permit determination of Ea. (Figure 8 drew from X-axis and Y axis data of Table 10).