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OPTIMIZATION OF β-D-GALACTOSIDASE RAPID ENZYME ASSAY USING *ESCHERICHIA COLI* ATCC 8739

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SUMMARY

The bacterial enzyme, β-D-galactosidase, catalyzes the breakdown of the complex sugar lactose into its components - galactose and glucose- simple sugars. The glycolysis of lactose by β-D-galactosidase is a point of attack for studies of the biochemical problem in disaccharide utilization and the genetic basis of enzyme constitution β-D galactosidase. The aim of the present study was to focus on optimization of a rapid enumeration method based on the enzymatic hydrolysis of 4-methylumbelliferyl-β-D-galactoside (MUGal) for toxicity test using *Escherichia coli* (*E. coli*) ATCC 8739 as a model. This rapid assay is based on the assumption that β-D-galactosidase is one marker for *E. coli* ATCC 8739. The enzymatic activity of *E. coli* ATCC 8739 was measured in a 25-minute assay. The effects of pH, temperature, nutrition, and substrate concentrations on enzyme activity were investigated. The enzyme β-D-galactosidase was shown to be induced with the inducer isopropyl-beta-D-thiogalactopyranoside (IPTG). The high level of production of β-D-galactosidase was found at bacteria incubated in tryptic soy broth without dextrose with IPTG. The optimum pH and temperature for β-D-galactosidase activity of *E. coli* ATCC 8739 was found to be 6.8 and 44.5 ^{o}C, respectively. The enzyme activity detected increased when raising the concentrations of the substrate (MUGal). The good linear correlation between logarithms of enzyme activities and CFU showed following supported the use of this method for toxicity experiments. Enzymatic methods and reference plate counts were significantly correlated. This method is sensitive, simple to perform and can be used as an alternative for traditional methods in detecting cell viability.

Keywords: *β-D-galactosidase, E. coli, enzyme assay, MUGal, IPTG, enzymatic activity*

INTRODUCTION

Indicators of pollution (e.g. coliform, fecal coliforms, and *Escherichia coli* (*E. coli*)) are used for monitoring the safety of water (Rompre *et al*., 2002). Some rapid techniques for detection of fecal coliforms and *E. coli* are based on enzymatic hydrolysis of fluorogenic substrates for β-D-galactosidase (Eaton *et al*., 1995; Manafi *et al*., 1991). Rapid detection is the accuracy approach for microbiological testing applications. These methods were developed to protect the public health that can bypass the need for time-consuming isolation procedures prior to identification. The elapsed time of assays is less than 6 h instead of 24 to 72 h for culture-based methods (George *et al*., 2000).

Rapid methods are based on one of the markers for fecal coliforms and *E. coli*, β-D-galactosidase. The β-D-galactosidase assays (whole cells) can detect both the cultivable and non-cultivable bacteria. Thus, the rapid assays can quantify bacteria *in situ* for assessment of the bacterial viability. β-d-galactosidase has been found in several microorganisms, including Gram-negative bacteria (e.g., strains belonging to the *Enterobacteriaceae*, *Vibrionaceae*, *Pseudomonadaceae*, and *Neisseriaceae*), several Gram-positive bacteria, yeasts, protozoa, and fungi (Tryland, Fiksdal, 1998). The β-D-galactosidase assays can apply in numerous experimental purposes, e.g. toxicity tests. In this study, the developed strategy is based on using the compound 4-methylumbelliferyl β-D-galactoside (MUGal), which is hydrolyzed rapidly by the action of β-D-galactosidase enzyme of *E. coli* ATCC 8739 to yield a fluorogenic product that can be quantified and directly related to the number of *E. coli* cells present in water samples. *E. coli* ATCC 8739 has also been used extensively in laboratories for over 70 years (Archer *et al.,* 2011); more recently, it has been used widely in toxicity test

The objective of this study was to optimize the environmental aspects on the galactosidase enzyme

assays of *E. coli* ATCC 8739. The fluorogenic substrate chosen for this study was 4-methylumbelliferyl- β-D-galactoside. Based on this assay, the viability of bacterialcell can be detected rapidly for toxicity tests or the others laboratorial purposes.

MATERIALS AND METHODS

Materials

4-methylumbelliferyl-β-D-galactoside (MUGal), isopropyl-beta-D-thiogalactopyranoside (IPTG), and Xgal were purchased from Sigma Chemical Company (MO, USA). *E. coli* ATCC 8739 strain was obtained from the Global Bioresource Center (American Type culture collection, VA, USA).

Methods

Enumeration of culturable E. coli ATCC 8739

The strain was plated onto nutrient agar and incubated overnight at 37ºC. A single colony from the overnight incubation was used to prepare a liquid culture for experiments. This liquid culture was incubated overnight at 37ºC with continuous orbital shaking at 1500 rpm (Lab-line® orbit shaker 3590, IL, USA) and was harvested at a stationary growth phase (based on a predetermined growth curve). The cell suspension was centrifuged at 4500 *g* for 15 min and re-suspended in a phosphate buffered saline (PBS) solution twice before use in experiments.

Galactosidase assay

The galactosidase enzymatic assay of whole cells was modified from a protocol by Fiksdal et al. (1994). The sample from the incubation experiment was filtered through a 0.2 μm pore-size, 47 mm diameter polycarbonate filter (Millipore Isopore Polycarbonate membrane filters, Fisher scientific, PA, USA). The filter with retaining was placed in a 250 ml flask containing 20 ml of a PBS solution supplemented with MUGal, 0.2 g/l sodium dodecyl sulfate, and 0.1% nutrient broth. The flask was incubated in a reciprocal shaking bath (Thermo electron corporation 2879, OH, USA). The fluorescence intensities of sample aliquots (1,0 milliliter of sample and 40 μl of 10 M NaOH) were measured every 5 minutes for 25 minutes with a spectrophotometer (Synergy HT, Biotek, VT, USA) at an excitation wavelength of 360±20 nm (10 nm slit width) and emission wavelength of 440±20 nm (20 nm slit width).

Construction of the standard curve

The fluorometer was calibrated by using 100% of fluorescence intensity of methylumbelliferone (MUF) concentration from 50 to 30000 nmol/l. The measured fluorescence intensity was converted to MUF concentration. The enzyme activity was determined by a least-square linear regression of the fluorescence versus time based on a plot between MUF released per minute (George *et al*., 2000).

Effect of pH and temperature on β-galactosidase enzymatic assay

Bacteria were inoculated in liquid culture and harvested by filtering before testing the enzyme activity. To find out the influence of temperature on enzyme assay, 250ml flasks continuing 20ml PBS supplemented with 0.5 g/l MUGal containing the filter with retaining were incubated in a reciprocal shaking bath at different temperatures: 22.5 (room temperature), 37^{o}C, and 44.5^{o}C. The effect of pH on the enzyme activity was determined by adjusting the medium with different pH values ranging 5, 6, 6.5, 6.8, 7, 7.2, 8, and 8.5. A sample aliquot was collected at 5, 10, 15, 20, and 25 minute intervals.

Influence of different MUGal concentrations

To determine effects of the substrate concentrations on detecting enzyme assay activity, a range of MUGal concentrations was prepared including 50, 100, 200, 300, 500, 1000, and 1500mg/l. A control (no MUGal) was operated in parallel. The flask was incubated at 44.5^{o}C.

Influence of different culture on the galactosidase production

To find out the suitability of culture for galactosidase production, bacteria were inoculated in three different media *viz*., tryptic soy broth, tryptic soy broth without dextrose, and triptic soy broth without dextrose supplemented with IPTG before testing the enzyme activity. The enzyme activity was measured at 5, 10, 15, 20 and 25-minute intervals for enzyme production.

Comparision of viable plate count (VPC) and galactosidase enzyme assay for bacterial viability

One colony of *E. coli* ATCC 8739 was inoculated in liquid culture (tryptic soy broth without dextrose supplemented with IPTG) at 37^{o}C. The sample aliquots were collected to determine cell densities by serial dilution and spread-plate and galactosidase enzyme assay at 0, 12, 24, 36, 48, 60,

90, 120, 150, 180, 210, and 240 hour intervals. The enzyme activity was correlated with the estimated cell numbers.

Statistical analysis

Analysis of variance (ANOVA), *t*-test at 5% significant level, Regression models, and Guassian relation were performed for data analysis. The PHStat Program of the Microsoft Excel version 12.0 and Graphpad Prism 5 were used for statistical analysis.

RESULTS AND DISCUSSION

Optimization of the β-D-galactosidase assay was carried out at various concentrations of MUGal, temperatures, pH, and media formulations. In this study, the β-D-galactosidase activities of *E. coli* ATCC 8739 at 44.5^{o}C, 37^{o}C, and 22.5^{o}C (room temperature) were compared. In general, the β-D-galactosidase activity depended on temperature (Figure 1). The maximum activity was exhibited at 44.5^{o}C. Consistent with the results of previous study (Tryland, Fiksdal, 1998; Caruso *et al*., 2002), enzyme activity rate increased when the temperature was raised from 22.5^{o}C (room temperature) to 44.5^{o}C and showed stable enzyme activity at 44.5^{o}C. Additionally, enzyme activity was stable when increasing temperature. It was the result of PBS supplemented with NB that supported an enzyme activator and enhanced thermal stability of β-D-galactosidase (Tryland *et al*., 1998).

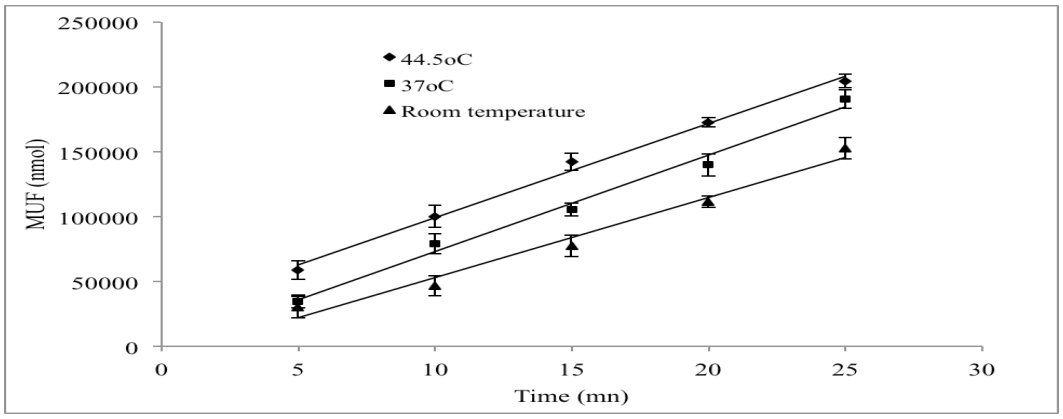

Figure 1. Influence of different temperature on enzyme activities of *E. coli* ATCC 8739.

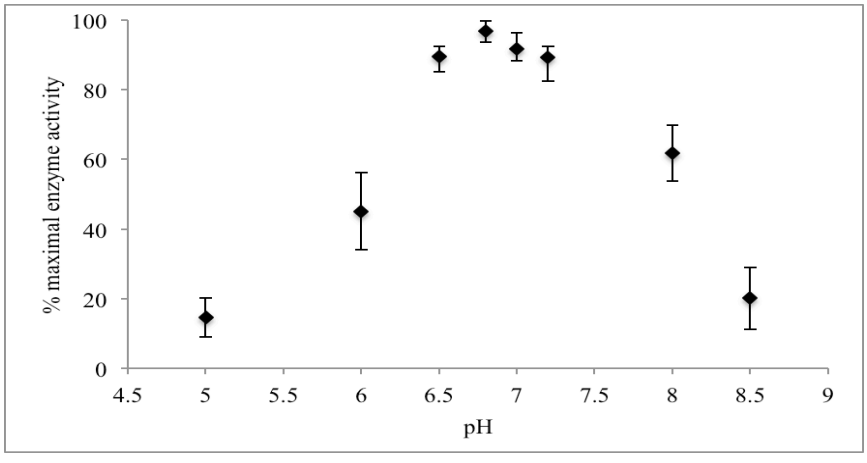

Figure 2. Influence of different pH on enzyme activities of *E. coli* ATCC 8739.

Enzyme activities were measured at various pH levels (Figure 2). As expected, the Gauss curves fitted to the data. Among the different pH levels tested, pH 6.8 was found optimum for galactosidase activity of *E. coli* ATCC 8739. It is provided that the pH optimum for growing of this strain is 6.8 ± 0.2. Thus, the enzyme production reached maximum when the cells were incubated in the proper pH level. In Figure 2, it is clear that pH stability profile of galactosidase ezyme is wide ranging from 6.4 to 7.3. Further experiments were conducted at pH 6.8.

When assessing the effect of MUGal concentration on β-D-galactosidase activity, as expected, Michaelis-Menten relationships were obtained. Enzyme activity was observed to increase with the increase of the concentration of the substrate up to 500 mg/l, with little increase in activity observed at higher concentrations of substrate (Figure 3). A routine substrate concentration of 500 mg/l was therefore selected for further experiments. This concentration allowed the measurement of enzyme activity close to the maximum.

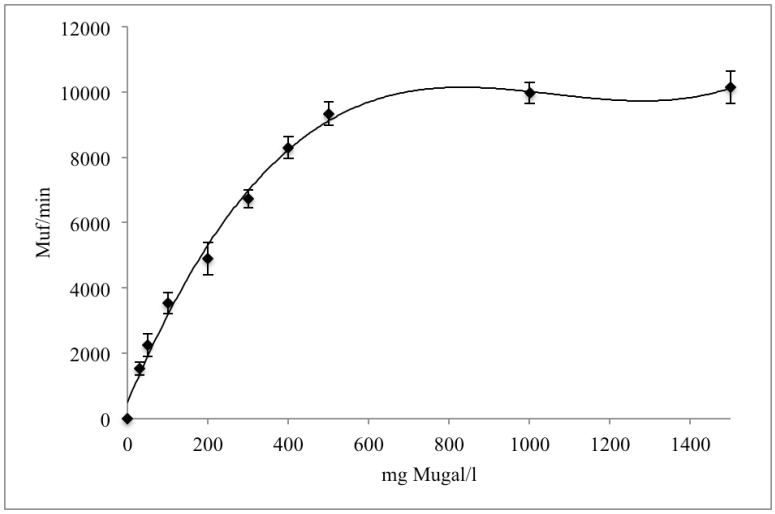

Figure 3. Influence of MUGal concentrations on enzyme activities of *E. coli* ATCC 8739.

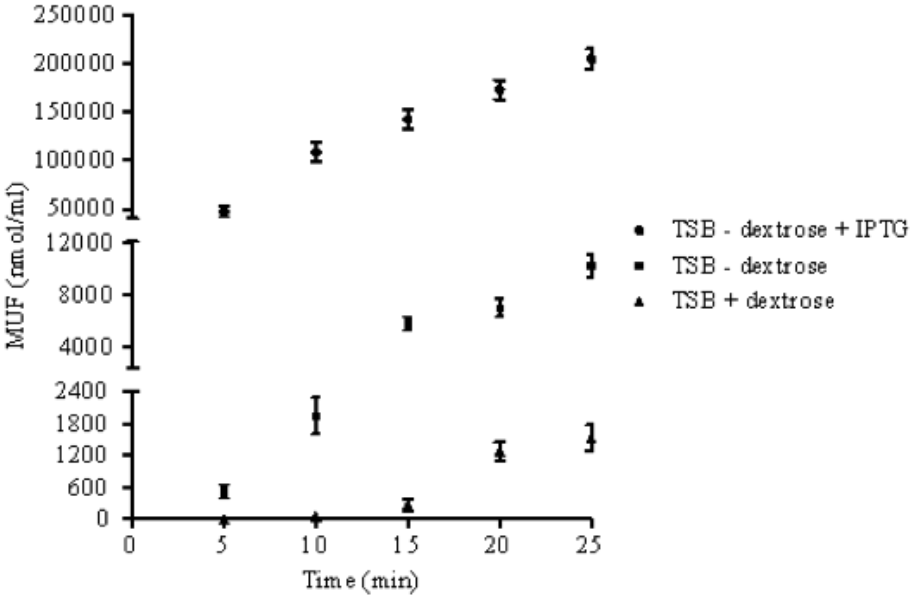

Figure 4. Influence of different nutrient media components on the enzyme activities of *E. coli* ATCC 8739.

Three nutrient media formulations were tested to determine the optimum media to use in experiments: these included tryptic soy broth, tryptic soy broth without dextrose, and tryptic soy broth with added IPTG but without dextrose. The significant difference in enzymatic activity was observed. The data (Figure 4) show that tryptic soy broth with added IPTG without dextrose resulted in the greatest enzyme activity. Additionally, β-D-galactosidase is known as an inducible enzyme (Herzenberg, 1959, Pardee, Prestidge, 1961). This is obvious evident that β-D-galactosidase released by *E. coli* ATCC 8739 was induced by IPTG. Therefore, we continue using the media added IPTG for incubating *E. coli* ATCC 8739.

Based on preliminary studies, bacteria incubated in tryptic soy broth without dextrose with added IPTG were harvested and washed twice before testing. The concentration of MUGal was adjusted to 500 mg/l. Enzyme activity was measured at 44.5^{o}C, pH 6.8. Using this approach, a standard curve was constructed using varying concentrations of *E. coli* (Log_{10} CFU/ml). Figure 5 shows that log-transformed enzymatic activities and log-transformed plate counts were well correlated. (Correlation coefficients of r = 0.903). The good linear correlation between logarithms of enzyme activities and CFU showed following supported the use of this method for toxicity experiments (Figure 5).

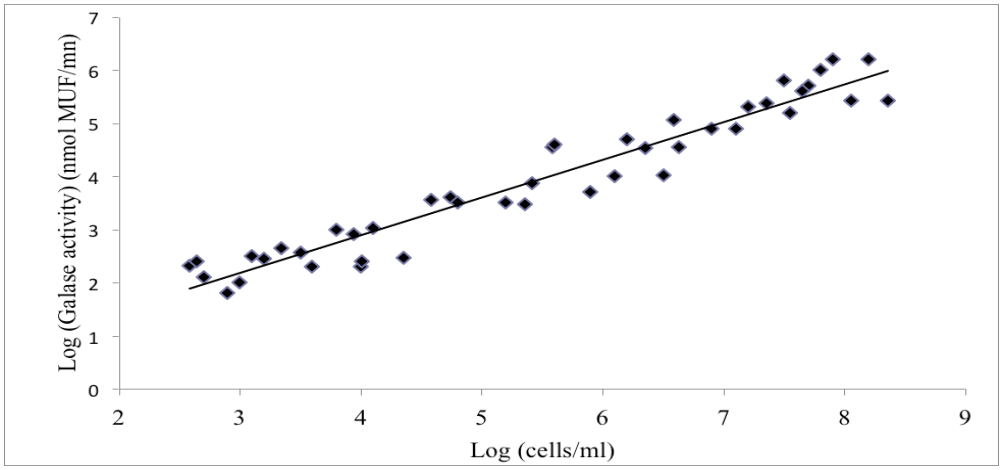

Figure 5. Relationship between β-D-galactosidase activity and total bacteria (CFU) of *E. coli* ATCC 8739.

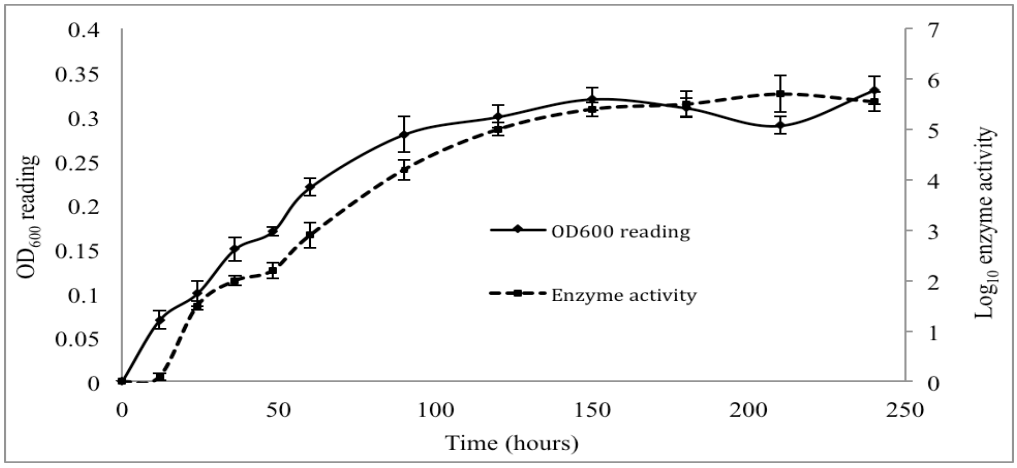

Figure 6. Growth curves using β-D-galactosidase activity and optical density (OD_{600}) of *E. coli* ATCC 8739.

The growth profile of *E. coli* detected by the plate count method, and enzyme assay showed in the figure 6. The enzyme activity was observed to start soon after beginning of incubation. The β-D-galactosidase went along well with the result of enumeration by plate count. The enzyme production was greatest when the cell population accessed to the stationary phase. This study reports, the enzyme activity compared with the growth rate of *E. coli*, as estimated by an optical measurement. It showed that enzyme activity appeared to couple with growth.

CONCLUSIONS

The production of enzyme β-D-galactosidase in coliform has been exploited in a multitude of new test based on the detection of hydrolysis products of fluorogenic substrates. These tests are sensitive, simple, and rapid to perform. They are applied to detect the microbial contamination in water. In this study, we determined the optimum growth and measurement parameter for detecting viability of *E. coli* ATCC 8739 (whole cells) using for toxicity tests. We have reported that ideal media for β-D-galactosidase activity from *E. coli* ATCC 8739 was tryptic soy broth without dextrose supplemented with IPTG. Enzyme activity gained the maximum rate at 44.5^{o}C, pH 6.8, in a test using 500mg/l of substrate, MUGal. The enzyme secretion is growth associated with the cell population. This supports the use of this method to access the cell viability in toxicity tests.

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TỐI ƯU HOÁ KỸ THUẬT TEST NHANH ENZYME β-D-GALACTOSIDASE SỬ DỤNG VI KHUẨN *ESCHERICHIA COLI* ATCC 8739

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Trường Đại học Đà Lạt

TÓM TẮT

β-D-galactosidase là enxyme xúc tác phản ứng thủy phân liên kết β-1,4-galactoside. Đây là một enzyme có

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nhiều ứng dụng trong công nghệ thực phẩm, sinh học, kỹ thuật và môi trường. Một phương pháp test nhanh dựa trên quá trình thủy phân enzyme của hợp chất 4-methylumbelliferyl-β-D-galactoside (MUGal) đã được tối ưu hóa trong nghiên cứu này nhằm áp dụng vào các thử nghiệm độc học có sử dụng vi khuẩn *Escherichia coli* (*E.coli*) ATCC 8739 như một đối tượng nghiên cứu. Phương pháp test nhanh này được dựa trên giả định rằng β-D-galactosidase là một biomarker của vi khuẩn *E. coli* ATCC 8739. Hoạt tính enzyme của vi khuẩn *E. coli* ATCC 8739 được kiểm tra trong các thử nghiệm kéo dài 25 phút. Trong bài báo này, chúng tôi đánh giá hoạt tính của enzyme, ảnh hưởng của pH, nhiệt độ, dinh dưỡng, nồng độ cơ chất. Enzym β-D-galactosidase được xúc tác bởi chất cảm ứng isopropyl-beta-D- thiogalactopyranoside (IPTG). Hoạt động của enzym β-D-galactosidase từ vi khuẩn *E. coli* ATCC 8739 được nuôi cấy trong môi trường tryptic soy mà không có dextrose nhưng được bổ sung IPTG là cao nhất. pH và nhiệt độ tối ưu cho hoạt động của enzym β-D-galactosidase tương ứng là 6,8 và 44,5^{o}C. Các hoạt động của enzym tăng lên khi tăng nồng độ chất nền (MUGal). Kết quả cũng cho thấy có mối tương quan tuyến tính giữa logarit của hoạt tính enzyme và CFU. Đây là dữ liệu quan trọng minh chứng cho khả năng sử dụng phương pháp này cho các thí nghiệm độc học. Kết quả thu được từ kỹ thuật test nhanh enzyme và phương pháp cổ điển để điều tra mức độ sống của tế bào vi khuẩn có mối quan hệ mật thiết với nhau. Do đó, phương pháp này có thể được sử dụng như là một phương pháp thay thế cho các phương pháp truyền thống để xác định tỷ lệ sống và sinh trưởng của tế bào vi khuẩn *E. coli*.

Từ khoá*: β-D-galactosidase, E. coli, kỹ thuật enzym, MUGal, IPTG, hoạt tính enzyme*