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Lactase from Clarias Gariepinus and its Application in Development of Lactose Sensor

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Abstract: A natural source of lactase enzyme was reported for the first time from an omnivorous cat fish, *Clarias gariepinus*. The lactase enzyme has been partially purified from catfish intestine using FPLC. The enzyme had specific activity 6.75 units mg^{-1} with an overall recovery of 80%. The enzyme was characterized and kinetic properties such as K_m (4.8mM), Vmax (11.66 μmoles $min^{-1}mg^{-1}$), optimum pH (7.2 – 7.5) and thermal inactivation have been studied. The enzyme was stable on storage at 4 ^oC with only 5% loss in enzyme activity in 120 days. The energy of activation for thermal inactivation of catfish lactase was 23 K cal mol^{-1}. The partially purified enzyme was used for the fabrication of lactose biosensor. *Copyright © 2007 IFSA.*

Keywords: Catfish lactase, Clarias gariepinus, Lactose, Lactose intolerance

1. Introduction

Lactase (β-galactosidase) is a metalloenzyme exhibiting broad substrate specificity. The specificity of the enzyme is confined to the sugar moiety and the anomeric character of the linkage but not to a particular a glycon [1]. Lactase catalyzes the hydrolysis of lactose to glucose and galactose. β-galactosidases are widespread in microorganisms, animals and plants [2, 4]. The enzyme lactase in humans and other mammals is found primarily in the intestine (jejunum). It is located on the luminal side of the cell membrane of villi cells. The enzyme has been reviewed in detail by Wallenfels and Weil [1]. Lactase may be used for determining lactose in food, blood and other biological fluids. Another important application of lactase is in food processing such as in the treatment of milk and milk products for lactose intolerant people.

The molecular weight of lactase from different sources varies from 67-630 KD [2]. β-galactosidase from *E. coli* is tetrameric, being composed of four identical subunits of 135 KD each with an independent active site [5]. The enzyme is fragmented into small peptides and the amino acid analysis indicates approximately 1170 residues per subunit [6, 7]. The optimum pH for the enzyme is 6-8 [8]. Monovalent cations have a stimulatory effect on β-galactosidase [9]. The alcohols, methanol, ethanol, iso-propanol, and n-propanol, (at 5% concentration) increase the rate of o-nitrophenyl β-D-galactopyranoside cleavage [10, 11]. The enzyme is protected against heat-inactivation by 5-phosphorylribose 1-pyrophosphate in the presence of β-mercaptoethanol [12]. In the absence of Mg^{2+} ions, β-mercaptoethanol causes dissociation of enzyme into subunits [13-15]. The enzyme is stable for 4 - 6 months when stored at below 5 °C. Half of its activity is lost in 40 min within the presence of stabilizers Mg^{++} and β-mercaptoethanol. Without these stabilizers the activity is lost within 10 min. The enzyme is fairly stable up to 40°C at pH 6-8 and the stability decreases sharply below pH 6 and gradually above pH 8. Galactose is found as a competitive inhibitor with a K_i of 9.4 $x10^{-3}$ μm, whereas, glucose is a non-competitive inhibitor < 10 mM concentration [16].

Different disaccharide hydrolyzing enzymes are reported in fish [17, 18] but the literature survey reveals that lactase of fish origin has not been studied in detail. Information regarding enzyme kinetics and other parameters lactase pertain to mainly mammals, plant and microorganisms. The present work deals with purification of lactase enzyme and its application in development of lactose biosensor.

2. Materials and Method

2.1. Chemicals

Anion exchanger Q-sepharose (fast flow) was from Amersham Biosciences, USA. Acrylamide, bis-acrylamide, galactose oxidase, TEMED, β-mercaptoethanol, Formvar (Polyvinyl formal resin), TRIS, phenylmethylsulfonyl fluoride (PMSF) and dimethylformamide (DMF) were obtained from Sigma-Aldrich Chemicals, USA. Coomassie blue, X-gal and ONPG were purchased from Sisco Research Laboratories, India. All other chemicals used were of analytical reagent grade from Qualigens, India. Synthetic milk Zerolac (lasctose/galactose free) was purchased from local medical store market. Amperometric measurements were conducted by using Aqualytic dissolved oxygen meter, Model OX-24, Germany.

2.2. Immobilization of Lactase and Galactose Oxidase

Lactase from *Clarias gariepinus* (60 units) and galactose oxidase (150 units) were dissolved in 200 μl of 100 mM sodium phosphate buffer, pH 7.0. Formvar (40 mg) was dissolved in 1 ml of chloroform/ethylene dichloride mixture (1:1 v/v) in a tube. The enzyme solution and formvar solution were mixed quickly and spread evenly on to a clean glass plate at room temperature for 1 h. A thin membrane was formed on the surface of glass plate. The lactase and galactose oxidase immobilized formvar membrane (12X5 cm^2) was then carefully scrapped from the glass plate and washed several times with water to remove unbound enzymes. The immobilized enzyme membrane was stored at 4 ^oC. The immobilized enzyme membrane was attached by o-ring at the sensing tip of the electrode of the dissolved oxygen meter.

2.3. Collection and Care of Fish

The Catfish *(Clarias gariepinus),* obtained from local market and were maintained at a temperature of 25 ± 1 ^{o}C and a lighting schedule of 12 h of light. Water in the plastic pools was renewed daily with stored tap water adjusted to laboratory temperature.

2.4. Assay of Lactase Activity

The enzyme activity was determined according to the method of Craven et al. [19] by monitoring the rate of increase of absorbance at 410 nm, due to the formation of ONP a yellow coloured product in Beckman spectrophotometer. Reaction was started by adding 100 µl of diluted enzyme solution to 1 ml assay buffer (100 mM sodium phosphate buffer, pH 7.2) containing 2.5 mM ONPG and 0.3 mM magnesium chloride at 30 ^{o}C. The increase in absorbance at 410 nm was recorded at 15 sec interval and the rate of reaction was obtained graphically (from the zero time slope). The extinction coefficient (ε) of ONP was 3.5 x 10^{3} M^{-1} cm^{-1}. An enzyme unit has been defined as the amount of enzyme that causes the hydrolysis of one µmole of o-nitrophenyl-β-D-galactopyranoside (ONPG) in one minute at pH 7.2 and 30 ^{o}C under specific test conditions. The specific activity of the enzyme is expressed as units of lactase per mg protein. Protein was estimated according to the method of Lowry et al. [20].

2.5. Screening of Tissues for Lactase Activity

Fishes were collected and immediately killed by decapitation. Brain, stomach, intestine and muscle were removed and placed in humid chamber at 4 ^{o}C. The tissues 2 g of each was cut into small pieces and transferred in separate microtubes containing 15 µl of 10 mM X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside) solution in DMF (dimethylformamide). The tubes were incubated in dark at 30 ^{o}C for 25 min. Blue-green colour developed in all the tubes with different intensities except in muscle. The maximum intensity was observed in brain followed by alimentary canal. Due to small size and weight of brain, it was not possible to select it as a source for isolation of β-galactosidase. The activity of enzyme was seen throughout the alimentary considering the presence of enzyme and large proportion pyloric region of the intestine was selected for isolation and purification of β-galactosidase. Presence of β-galactosidase in intestine accounts for metabolism of lactose in catfish.

2.6. Isolation of β-galactosidase

The following steps were carried at 0-4 ^{o}C unless stated otherwise.

2.6.1. Crude Extract

The intestine (50 g) was collected from freshly decapitated fish and cleaned with distilled water. The segment of intestine was minced thoroughly and homogenized with 50 ml of 50 mM sodium phosphate buffer, pH 7.2 containing 1mM EDTA and 1 mM PMSF (protease inhibitor). The homogenate was then centrifuged at 24,000 g for 60 min in Sorvall 5B centrifuge. The pellet was discarded and the supernatant was dialyzed extensively against 10 mM Tris-HCl buffer, pH 8.0 to remove coloured impurities. Equilibrated crude solution was loaded on to the anion exchange Q-sepharose column.

2.6.2. Fast Protein Liquid Chromatography (FPLC)

The dialyzed crude solution (50ml) was applied to anion exchange Q-sepharose column interfaced with Pharmacia FPLC^{TM} system. The column was washed with 10 bed volumes of washing buffer (10 mM Tris-HCl, pH 8.0) until the absorbance at 280 was less than 0.01. The flow rate was maintained at 1.5 ml/min. The bound protein was eluted with a linear gradient of 0.5M sodium chloride in 20 mM Tris-HCl buffer, pH 8.0. The results are shown in Fig. 1. The maximum activity of β-galactosidase was eluted in fraction no. 45. The protein containing fractions showing β-galactosidase activity were pooled (28 ml) and dialyzed against several changes of 50 mM sodium phosphate buffer, pH 7.2 to remove sodium chloride. The dialyzed sample was lyophilized to concentrate the enzyme and the lyophilized powder was stored below 0 ^{o}C until further use.

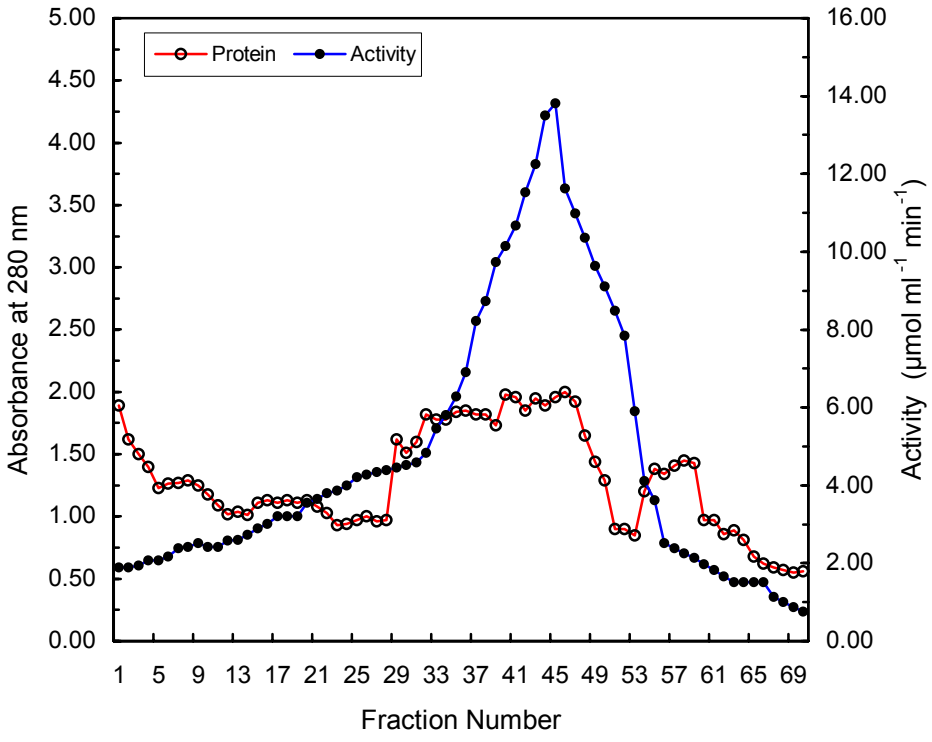

Fig.1. FPLC of lactase on anion exchange Q-sepharose column. Lactase activity (●) was estimated according to the method of Craven et al [19]. Protein absorbance at 280 nm (o) was recorded in Beckman DU64 spectrophotometer. Fractions 35-53 (approx 1.5 ml each) were pooled (28.5 ml) and lyophilized.

2.7. Enzyme Characterization

2.7.1. Gel Electrophoresis

Native polyacrylamide gel electrophoresis (PAGE) of the lyophilized β-galactosidase was performed at pH 8.3 as described by Reisfield et al [21]. Polyacrylamide gel electrophoresis in the presence of anionic detergent, sodium dodecyl sulphate (SDS) was also carried out at pH 8.0 in 10 % gel according to the method of Weber and Osborn [22].

2.7.2. Effect of pH

To check the stability lactase enzyme was incubated with the respective buffer at 30^{o}C for 10 min and enzyme assay was carried out as described above. The effect of pH on catfish β-galactosidase activity has been studied using 100 mM sodium phosphate buffers (pH 5.5, 6.0,6.5, 7.0, 7.5 and 8.0) maintaining high enzyme-saturating concentration of substrate (2.5 mM ONPG).

2.7.3. Effect of Temperature

The effect of temperature on lactase was assessed by carrying the enzyme assay at various temperature (20-55^{o}C). The enzyme solution was incubated at desired temperature for 5 min and aliquots (100 µl) were withdrawn and assayed according to the method of Craven et al [10].

2.7.4. Storage and Thermal Inactivation of Enzyme

The enzyme was stored in 100 mM sodium phosphate buffer, pH 7.2 at 25, 4 and -10 ^{0}C. Aliquots were withdrawn at different days and activity was measured as described earlier. Thermal inactivation of partially purified catfish β-galactosidase has been studied at 40, 45 and 50 ^{o}C. The enzyme solution, was maintained at the desired temperature and aliquots withdrawn at different intervals, were assayed for enzyme activity as usual.

2.7.5. Km and Vmax

Lineweaver-Burk plot was made at different concentrations of substrate (ONPG) in 100 mM sodium phosphate buffer, pH 7.2 at 30 ^{0}C and Michelis-Menten Constant (K_m) and maximum velocity (V_{max}) were estimated for lactase enzyme.

3. Results and Discussion

3.1. Purification of Lactase

A summary of purification of Indian catfish lactase from 50 g intestine tissue is shown in Table 1. The enzyme was purified 169 fold increase in specific activity with an overall recovery of about 80 %. The enzyme may be further purified to improve specific activity using affinity chromatography or other methods. Our objective was to purify lactase from a new natural source for the development of lactose biosensors for lactose intolerance people. Though, commercially available purified enzyme is mostly used for the enzymatic reactions in biosensors as well as in other biochemical reactions. However, partially purified enzyme lactase from *Clarias gariepinus* can also be used for the development of biosensors without any major hinderance in catalytic reaction [23, 24].

3.2. Ultraviolet Absorption Spectrum

The lyophilized catfish lactase shows a typical protein absorption in the UV region with maximum absorption at 278 nm and A_{280}/A_{260} ratio equal to 0.94. At one mg protein per ml concentration the solution shows an absorbance of 1.28 at A_{280}. The A_{280}/A_{260} ratio suggests that the enzyme has relatively low amount of bound nucleotides (4%).

Table 1. Purification of lactase from *Clarias gariepinus* intestine (50 g).

Step	Volume (ml)	Activity (units)	Protein (mg)	Specific activity (units/mg)	Fold Purification	Yield (%)
Crude extract	50	168	3950	0.04	-	100
FPLC	28	138	22	6.27	157	82
Lyophilized Powder* (20mg protein)	20	135	20	6.75	169	80

*Lyophilized powder of enzyme was dissolved in extraction buffer (1 mg protein/ml). Protein was estimated according to the method of Lowry et al [20]. The enzyme activity was estimated as described in materials and methods.

3.3. Polyacrylamide Gel Electrophoresis

The native gel electrophoresis of partially purified lactase showed more than one protein bands, suggesting that the enzyme is partially purified. However, the enzyme may be further purified using affinity chromatography to achieve single protein band. SDS gel electrophoresis of lactase shows more protein bands (four). Since the enzyme is partially purified, therefore to achieve exact subunits of lactase further purification will be required. Here, the partially purified enzyme may fulfill the requirement for development of lactose biosensors. Therefore, enzyme was not further purified. However, the enzyme may be later purified to achieve the homogeneity and subunit of catfish lactase.

3.4. Effect of pH on Lactase Activity

The pH profile of cat fish lactase is shown in Fig. 2. Maximum activity was observed at pH 7.2-7.5. The enzyme activity increases from pH 5.5 to 7.2 and then decreases sharply beyond pH 7.5. Thus, the pH of maximum activity (pH 7.2 - 7.5) represents the pH optimum of catfish lactase. This value is within the range of optimum pH reported for lactase isolated from other sources [2].

3.5. Effect of Temperature on Lactase Activity

The enzyme activity of lactase increases gradually from 20-47 ^{o}C and then decreases sharply at saturated concentration of artificial substrate ONPG (2.5 mM) in 100 mM sodium phosphate buffer, pH 7.5 (Fig.3). At higher temperature enzyme denatured faster than low temperature. Therefore, Lactase enzyme is always kept at lower temperature and assayed at room temperature to avoid denaturation of enzyme.

3.6. Storage of Catfish Lactase

The enzyme was fairly stable when stored in 100 mM sodium phosphate buffer, pH 7.5 at 4 ^{o}C. Only 5 % loss in activity was observed in 120 days. The enzyme is comparatively less stable when stored at 25 ^{o}C in 100 mM sodium phosphate buffer, pH 7.5. The loss of activity in this case is continuous and exponential with $t_{1/2}$ equal to more than 4 months (Fig. 4). The enzyme was more stable when stored as lyophilized powder below 0 ^{o}C (-10 ^{o}C). Therefore, the enzyme was routinely stored as lyophilized powder below 0 ^{o}C or in solution at 4 ^{o}C.

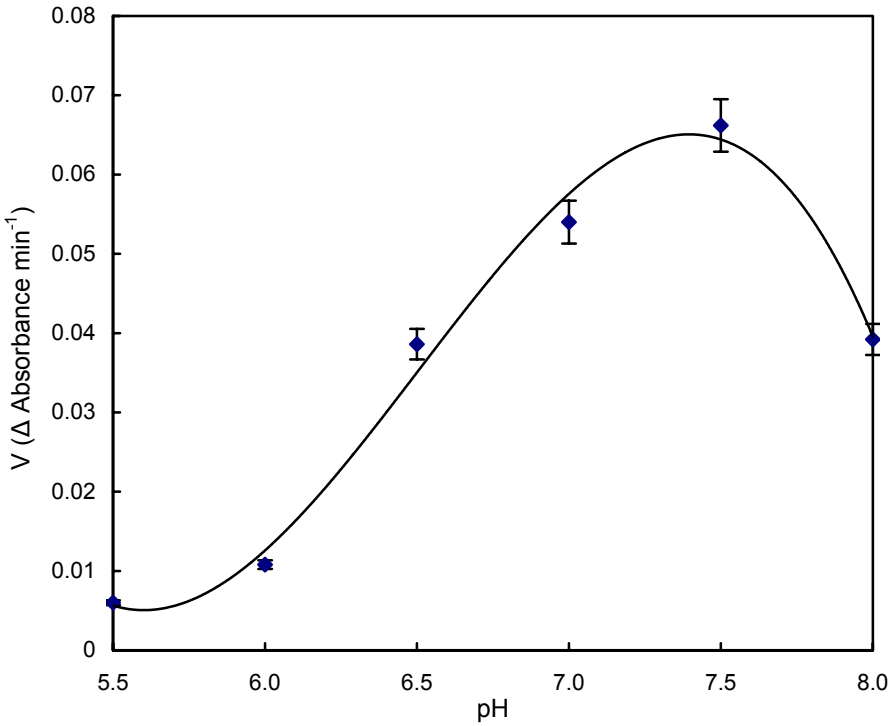

Fig.2. Effect of pH on catfish lactase activity in 100 mM sodium phosphate buffers at 30 °C. The enzyme concentration in each case was 30 μg/ml. The concentration of ONPG was 2.5 mM.

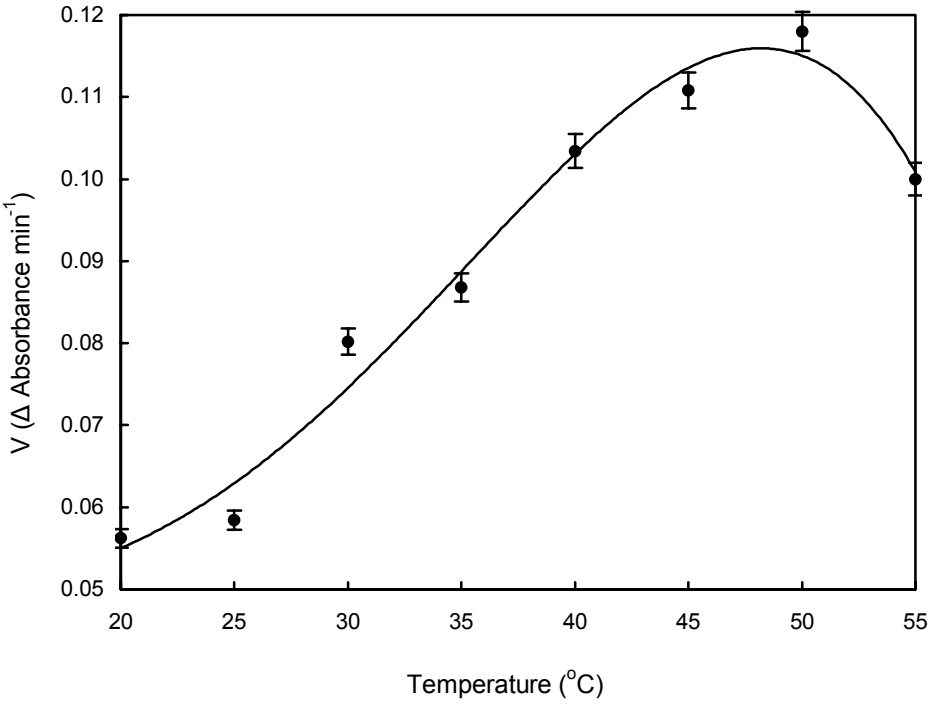

Fig.3. Effect of temperature on catfish lactase in 100 mM sodium phosphate buffer, pH 7.5. The enzyme concentration in each case was 30 μg/ml. The concentration of ONPG was 2.5 mM.

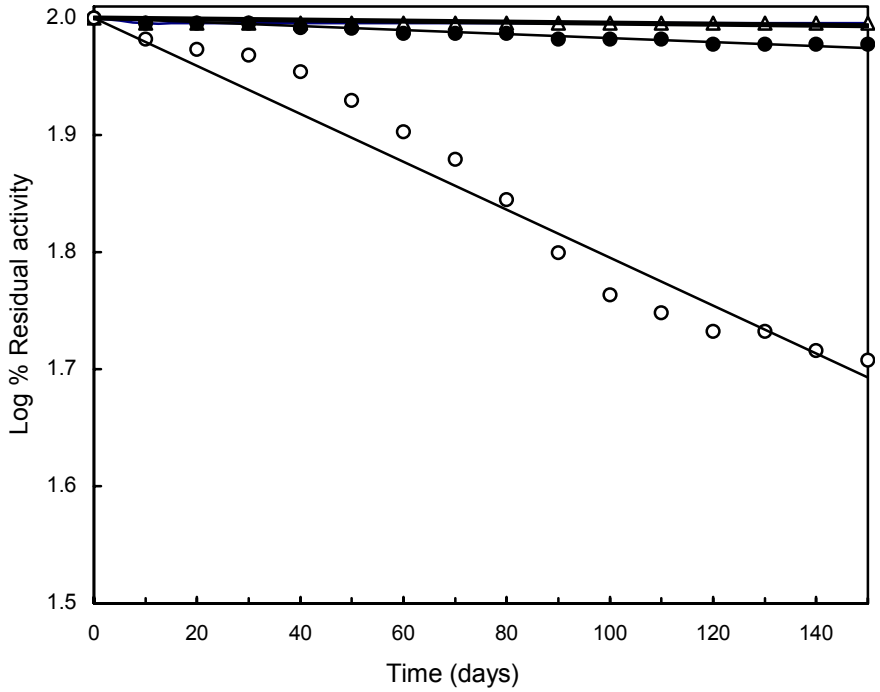

Fig.4. Semi log-plot for the loss of catfish lactase activity on storage in 100 mM sodium phosphate buffer, pH 7.2 at 25 ^{o}C (o) and 4 ^{o}C (●) and as lyophilized powder at –10 ^{o}C (Δ). The concentration of protein in each case was 30 μg/ml. Aliquots were withdrawn at different days and assayed for activity.

3.7. Thermal Inactivation of Catfish Lactase

Thermal inactivation of cat fish lactase is shown in Fig.5. Thermal inactivation of lactase follows simple first order kinetics (single exponential decay). The values of first order rate constants of inactivation at 40, 45 and 50 ^{0}C are shown in Table 2.

Arrhenius plot (log $k_{inactivation}$ versus 1/T of the data of Table 2, the energy of activation for thermal inactivation of catfish lactase was found to be 23 K cal mol^{-1}.

Table 2. First order rate constants for thermal inactivation of catfish lactase.

Temp (^{o}C)	Temp (K)	1/T (K^{-1})	Rate constant (k) (min^{-1})	ln k (min^{-1})
40	313	3.195 x 10^{3}	0.0082	-4.8036
45	318	3.145 x 10^{3}	0.0346	-3.3639
50	323	3.096 x 10^{3}	0.1386	-1.9761

k = 0.693 / $t_{1/2,}$ T = Temperature (Kelvin), ln k = natural logarithm of

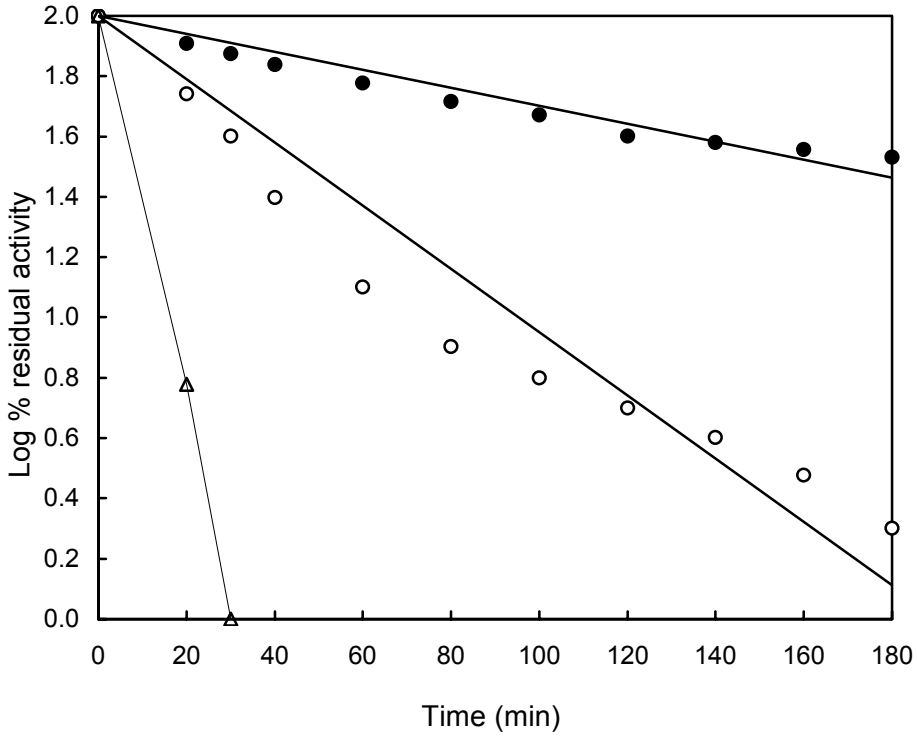

Fig.5. Thermal inactivation of catfish lactase in 100m M sodium phosphate buffer, pH 7.2 at 40 (●), 45 (o) and 50 ^{o}C (Δ). The enzyme solution was rapidly brought to the desired temperature (± 0.1^{o}C). Aliquots were withdrawn at different intervals and assayed for the residual enzyme activity. The protein concentration in each was 30 μg/ml.

3.8. Determination of K_m and V_{max}

The K_m and V_{max} values were calculated from the double reciprocal plot (Lineweaver-Burk plot), 1/V versus 1/[s] by varying ONPG concentrations (Fig.6). The K_m of ONPG for catfish lactase was found to be 4.8 mM, which is similar to that reported β-galactosidase from different sources [2]. The V_{max} was found to be 11.66 μmoles min^{-1} mg^{-1}.

3.9. Response Measurements

The fabrication of lactose biosensor was made as shown in Fig.7.The response (dissolved oxygen mg l^{-1} depletion) of the immobilized enzymes in a formvar membrane attached to an oxygen electrode (lactose sensor) at different concentrations of lactose in a 100 mM phosphate buffer of pH 6.5 as well as in lactose/ galactose free zerolac synthetic milk (10 g solid powder dl^{-1}water) was determined at 25 ^{o}C (Fig. 8). It showed linearity from 1 to 7 g dl^{-1} after which a limiting value of response was obtained. After measuring the response, the membrane was washed thoroughly with water and subsequently dried. The same electrode was used for testing the working stability (reusability) and the response was compared with a fresh membrane. It was observed that the membrane may be used 20 times, with 5-10 % loss in response (data not shown). Beyond this, there was severe loss (40-50%) in response. Lactose biosensor works only in the presence of lactose containing samples. If sample contains galactose contamination it gives false result. Therefore, lactose biosensor is not recommended for use in galactose containing samples especially in hydrolyzed milk.

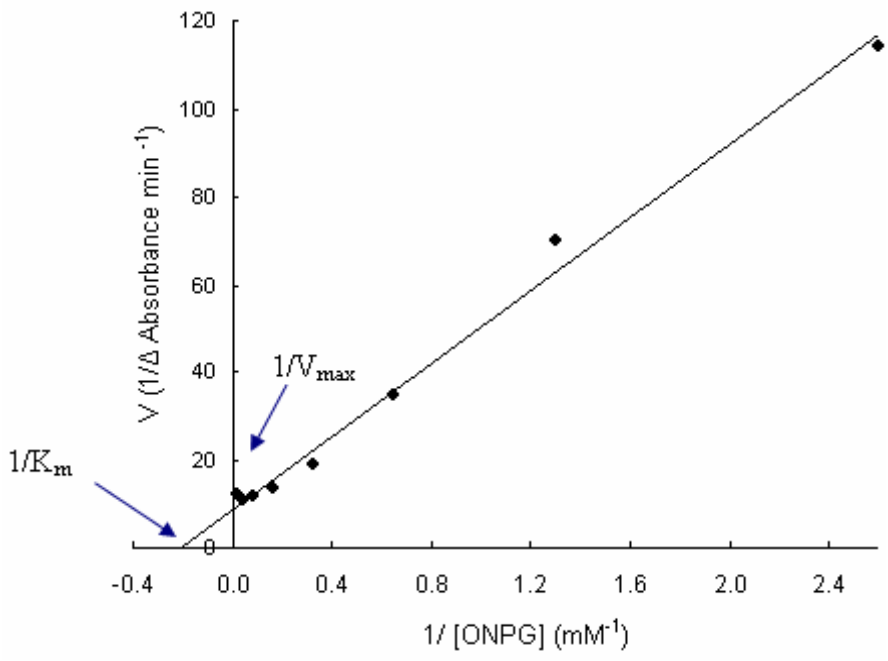

Fig.6. Lineweaver-Burk plot for the effect of substrate (ONPG) concentration on catfish lactase activity in 100 mM sodium phosphate buffer, pH 7.2 at 30 ^{o}C. The concentration of protein in each case was 30μg/ml. The assay mixture contains 0.2 units/ml β-galactosidase.

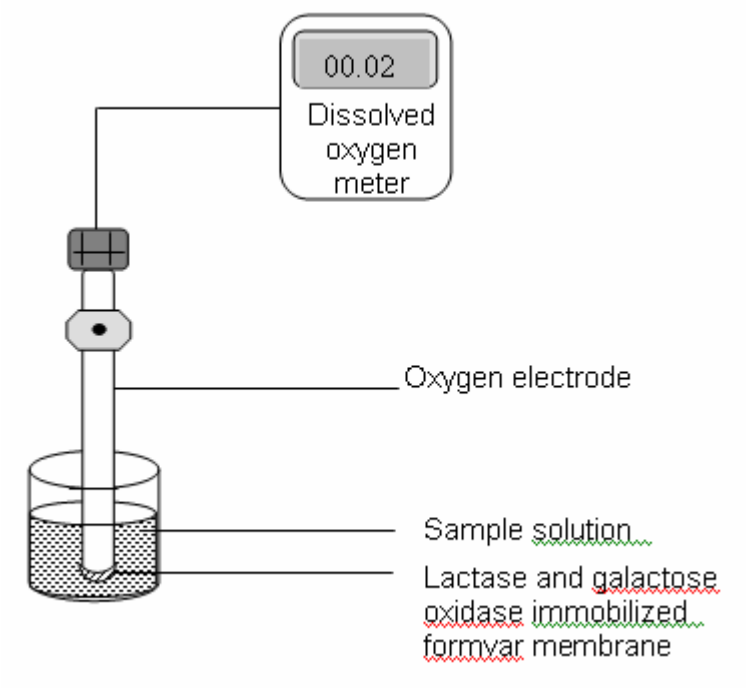

Fig.7. Schematic representation of lactose biosensor for estimation of lactose.

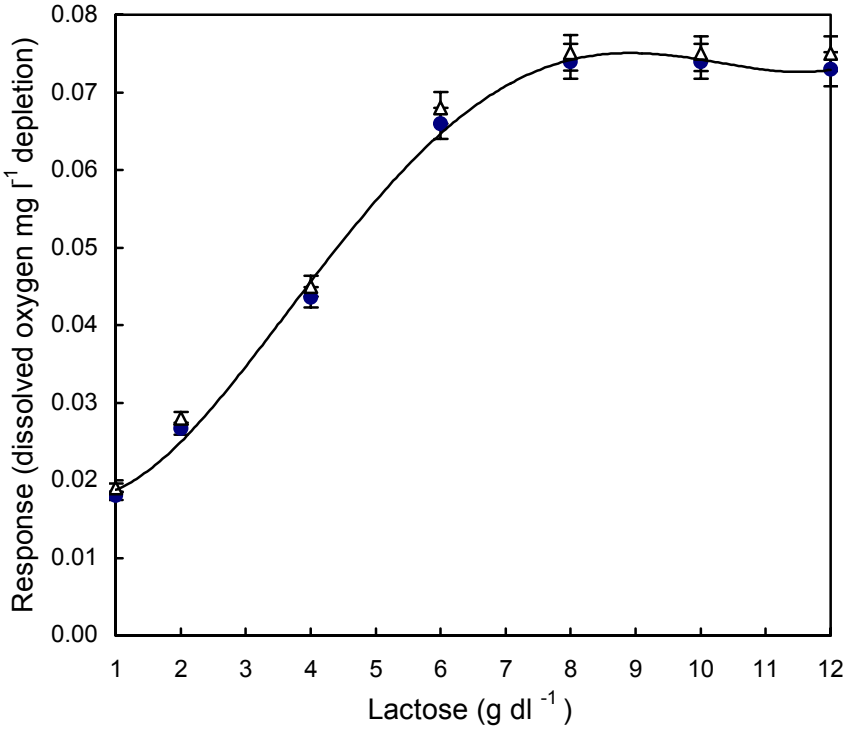

Fig.8. Response of the immobilized enzymes PVF membrane at different concentrations of lactose in 100 mM phosphate buffer, pH 6.5 (●) and in Zerolac synthetic milk (Δ).Five readings were taken at each measurement.

Conclusions

Lactase from *Clarias gariepinus* and galactose oxidase were immobilized in formvar membrane. The formvar membrane was attached to the electrode of a dissolved oxygen analyzer for the fabrication of a lactose biosensor. The biosensor can be used repeatedly, 20 times for the estimation of lactose and shows linearity for 1-7 g dl^{-1} lactose. Lactose biosensor works only in the presence of lactose containing samples. If sample contains galactose contamination it gives false result. Therefore, lactose biosensor is not recommended for use in galactose containing samples especially in hydrolyzed milk. For the detection of galactose in sample, galactose biosensor (without immobilized lactase) was already developed in our laboratory.

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Guide for Contributors

Aims and Scope

Sensors & Transducers Journal (ISSN 1726- 5479) provides an advanced forum for the science and technology of physical, chemical sensors and biosensors. It publishes state-of-the-art reviews, regular research and application specific papers, short notes, letters to Editor and sensors related books reviews as well as academic, practical and commercial information of interest to its readership. Because it is an open access, peer review international journal, papers rapidly published in *Sensors & Transducers Journal* will receive a very high publicity. The journal is published monthly as twelve issues per annual by International Frequency Association (IFSA). In additional, some special sponsored and conference issues published annually.

Topics Covered

Contributions are invited on all aspects of research, development and application of the science and technology of sensors, transducers and sensor instrumentations. Topics include, but are not restricted to:

- Physical, chemical and biosensors;
- Digital, frequency, period, duty-cycle, time interval, PWM, pulse number output sensors and transducers;
- Theory, principles, effects, design, standardization and modeling;
- Smart sensors and systems;
- Sensor instrumentation;
- Virtual instruments;
- Sensors interfaces, buses and networks;
- Signal processing;
- Frequency (period, duty-cycle)-to-digital converters, ADC;
- Technologies and materials;
- Nanosensors;
- Microsystems;
- Applications.

Submission of papers

Articles should be written in English. Authors are invited to submit by e-mail editor@sensorsportal.com 6-14 pages article (including abstract, illustrations (color or grayscale), photos and references) in both: MS Word (doc) and Acrobat (pdf) formats. Detailed preparation instructions, paper example and template of manuscript are available from the journal's webpage: http://www.sensorsportal.com/HTML/DIGEST/Submition.htm Authors must follow the instructions strictly when submitting their manuscripts.

Advertising Information

Advertising orders and enquires may be sent to sales@sensorsportal.com Please download also our media kit: http://www.sensorsportal.com/DOWNLOADS/Media_Kit_2007.PDF

Smart Sensors Systems Design

A five-day engineering course

5-9 November 2007, Barcelona (Spain)

SMARTSES
Marie Curie Chair (EXC) project

General Information

This course is suitable for engineers who design different digital and intelligent sensors, data acquisition, and measurement systems. It is also useful for researchers, graduate and post graduate students. Course will be taught in English.

Course Description

An advanced engineering course describes modern developments and trends in the field of smart sensor systems and digital sensors design.

After a general overview of data acquisition methods, modern smart, digital and quasi-digital sensors, smart systems details are discussed. A systematic approach towards the design of low-cost high-performance smart sensors systems with self-adaptation and self-identification possibilities is presented.

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Online Registration:
http://www.sensorsportal.com/HTML/SSSD_Course_2007.htm

Deadline for Registration:
25 October, 2007

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