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IMPROVED CATALYTIC ACTIVITY OF *ASPERGILLUS ORYZAE* β-GALACTOSIDASE BY COVALENT IMMOBILIZATION ON EUPERGIT CM

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ABSTRACT

In this study, *Aspergillus oryzae* β-Galactosidase (AOG) was immobilized onto Eupergit CM. By optimizing the immobilization conditions such as pH and molarity of immobilization buffer, enzyme/support ratio and duration of immobilization, 100.00% immobilization yield and 129.82% activity yield was achieved. The optimum temperature (55 °C) of free enzyme was not changed while optimum pH of free enzyme was shifted from 4.5 to 5.5 after immobilization. Kinetic constants for free and immobilized enzyme were also determined by using the Lineweaver-Burk plot. The K_m values of the free and immobilized enzymes were determined to be 307.7 and 234.2 g / L respectively, while the V_{max} values were determined to be 0.366 g D-Glucose / L.min and 0.415 g D-Glucose / L.min respectively. The operational and storage stabilities of immobilized enzyme were also studied. The activity of immobilized enzyme decreased to 99.3% after repeated twenty usage while decreased to 98.3% after fifteen days of storage. Further, the immobilized enzyme was used for the hydrolyzing the cow's milk lactose. By using the immobilized enzyme, the milk lactose was completely hydrolyzed in four hours. Consequently, immobilized AOG can be used in the industrial production of lactose-free cow's milk.

Key words: *Aspergillus oryzae;* β-Galactosidase; covalent immobilization; Eupergit CM; lactose-free cow's milk; lactose intolerance

INTRODUCTION

Lactose is a galactoside disaccharide linked to glucose by the β- (1,4) glycosidic linkage, and has a great importantce as the main calorie source of all mammals except sea lion. Absorption of lactose by intestine requires the hydrolysis of lactose to its components (glucose and galactose) with help of enzyme lactase (β-Galactosidase) (Deng *et al*., 2015). When the lactase enzyme is not synthesized in the organism, lactose passes through the large intestine without being digested in the small intestine and causes fermentation of digestive disorders, diarrhea and gas formation (Tunçbilek *et al*., 1973). This widespread health problem has been dissolved by consuming the lactose-free cow's milk.

β-galactosidases (EC.3.2.1.23) have various applications in the food and dairy industries. But, soluble enzymes have some disadvantages such as poor stability, difficulties in the removal of enzymes from the product, inability to use in continuous production processes, high product cost (Panesar *et al*., 2010). Therefore, it was needed to bring out their full potential as catalyst by developing new strategies for the enzyme stabilization. The stability and activity of the enzymes can be enhanced via immobilization. Furthermore, immobilized enzymes are easily separated from products and used in the continuous production processes in the industry. Thus product cost is decreased (Mateo *et al*., 2007).

Five different methods of immobilization have been developed according to the nature of the support used and the intended use: Cross-linking, adsorption, entrapment, encapsulation and covalent binding. Hundreds of natural and synthetic supports or matrixes have been used for immobilization of industrial enzymes. The selection of support and method for enzyme immobilization depends on the nature of the support, the simplicity of the method and the intended use of the enzyme (Klibanov, 1983). The covalent attachment is the more suitable method for all enzymes and for all applications since immobilized enzyme doesn't easily lose its activity.

Eupergit CM which carries epoxy groups on its surface and consists of porous acrylic microbeads is a commercial matrix developed for covalent immobilization of enzymes. Supports containing epoxy groups are excellent matrixes for immobilization of enzymes. They are very stable in wide ranges of pH and at high temperaure and compatible for all kinds of reactors. Enzymes can easily bind to these supports with the reactions between epoxy groups with sulfhydryl, hydroxyl and amino groups available in the side chain of amino acids depending on pH of the buffer used (Boller *et al*., 2002). Immobilization procedure is quite simple

and involves the reaction of Eupergit C beads with aqueous enzyme solution at the room temperature or at +4 ºC for 24-120 hours. Immobilization of enzymes onto epoxy activated matrix is affected by the amount of Eupergit C, pH and concentration of buffer used and duration of immobilization (Hernaiz and Crout, 2000; Martin *et al*., 2003). These conditions must be optimized to obtain highest possible activity yield in an enzyme immobilization study. Many of enzymes have been immobilized by using Eupergit C with up to 80 % activity yields. Eupergit CM has similar physicochemical properties with Eupergit C except for the amounts of epoxy groups available on the beads, the size and pore diameters. Immobilization of some enzymes has resulted in increased activity than free enzymes in the immobilization with Eupergit C (Torres-Bacete *et al*. 2000; Aslan and Tanrıseven, 2007; Zarcula *et al*., 2009) and with Eupergit CM (Aslan *et al*., 2013; Aslan *et al*., 2014).

AOG have been immobilized by using different supports and methods in a few studies (Huzjak *et al*., 1994; Mohy Eldin *et al*., 1999; El-Masry *et al*., 2000; Haider and Husain, 2007; Haider and Husain, 2009; Wu *et al*. 2010; Guidini *et al*., 2010; Freitas *et al*., 2011; Husain *et al*., 2011; Ansari *et al*., 2012; Ansari and Husain, 2012; Fischer *et al*., 2013; Ansari *et al*., 2015; Wahba, 2016). But according to our literature review, immobilization of AOG with Eupergit CM has not been studied. Therefore the aim of this study was to immobilize AOG by using the covalent binding method with the highest possible activity yield on Eupergit CM.

MATERIALS AND METHODS

Materials: AOG (Fungal Lactase) which have 7.7 IU/ g activity, is a commercial enzyme preparation, was provided as a gift by Bio-Cat (Troy, USA). Eupergit CM was a gift by Röhm and Haas (Darmstadt, Germany). UV-VIS Spectrometer (UV-6300PC) was purchased from VWR (Radnor, USA). pH meter (Hanna HI 2020 edge) was purchased from Hanna Instruments Ltd. (Bedfordshire, UK). The magnetic stirrer (Heidolph MR Hei-Standard) was purchased from Heidolph UK-Radleys (Shire Hill, UK). Pure water appliance (Mini Pure 1, MDM-0170) was purchased from MDM Co. Ltd. (Suwon-si, South Korea). Precision scale (Shimadzu-ATX224) was purchased from Shimadzu Corporation (Kyoto, Japan). Orbital shaking heated incubator (Mipro-MCI) was purchased from Protek Lab Group, professional laboratory solutions company (Ankara, Turkey). The vacuum pump (Biobase, GM-0.50A) was purchased from Biobase Biodustry Co., Ltd. (Shandong, China). Bovine Serum Albumin (BSA), sodium hydroxide, sodium dihydrogen phosphate, hydrochloric acid, lactose, sodium sulfite, phenol and D-glucose were purchased from Sigma-Aldrich (Taufkirchen, Germany). 3,5-dinitrosalicylic acid (DNS) was purchased from Alfa Aesar (Kandel, Germany). Sodium potassium tartrate (Rochelle salt) was purchased from VWR Prolabo Chemicals (Leuven Belgium). Sodium azide was purchased from Merck Millipore (Darmstadt, Germany). Milk was purchased from a local market.

Methods

Immobilization Procedure: Immobilization was performed by reacting 100 mg of Eupergit CM with 200 µL of AOG in 5 mL of sodium phosphate buffer (0.5 M, pH 4.5) at 25 ºC for 24 h in an incubator with gentle shook at 150 rpm (Degussa, 2007). After immobilization the beads were filtered and washed with 15 mL of sodium phosphate buffer (0.1 M, pH 4.5) and 15 mL distilled water on a sintered glass filter by suction under vacuum. After then, immobilized enzymes have been stored in 5 mL of sodium phosphate buffer (0.1 M, pH 4.5) in a refrigerator at +4 ºC until use.

Optimization of Immobilization Procedure: Immobilization of enzymes onto epoxy activated matrix is affected by the amount of Eupergit C, pH, and concentration of buffer used and duration of immobilization (Degussa, 2007). Optimum conditions for immobilization were reached by changing the conditions individually, (amount of Eupergit CM from 100 mg to 600 mg; pH from 3.5 to 5.5; buffer concentration from 0.5 M to 2.0 M; and duration of immobilization from 24 h to 120 h).

Protein Assay: The amounts of proteins present in the immobilization buffer before and after immobilization were determined by using Sigma Standard 3.1 ml Assay Protocol (Bradford Reagent (B6916) - Technical Bulletin, 2018) based on the Bradford Protein Assay Method (1976).

Determination of β-Galactosidase Activity: 5 mL lactose solutions (1 % w/v) prepared by using 25 mM sodium phosphate buffer (pH 4.5) were reacted with 200 µL free or 0.317 g immobilized AOG at 55 ºC for 60 min in an incubator with gently shaking. 200 µL of aliquots from the reaction mixture was added to 1800 µL of distilled water and boiled for 10 min to inactivate the enzyme. The amount of formed D-glucose was determined by measuring its absorbance using a UV spectrophotometer at 575 nm, according to the protocol of Wang (2018) based on the DNS method (Miller, 1959). One IU is defined as the amount of enzyme forming 1 µmol D-Glucose from lactose per minute, under optimum activity assay conditions.

Calculation of Immobilization and Activity Yields: The immobilization and activity yields were calculated by using following equations.

$$Immobilization\ Yield\ (\%) = \frac{Enzyme\ used\ for\ immobilization - enzyme\ retained\ in\ the\ filtrate}{Enzyme\ used\ for\ immobilization} x100 \quad (1)$$

$$Activity\ Yield\ (\%) = \frac{Activity\ of\ immobilized\ enzyme}{Activity\ of\ soluble\ enzyme\ used} x100 \quad (2)$$

Characterization of Free and Immobilized Enzyme

Effect of pH on Enzyme Activity: The effect of pH on enzyme activity was investigated by performing the activity assay for the 200 µL free or 0.317 g immobilized AOG with 1 % (w/v) lactose solutions, at different pH, at 55 ºC using the method described by Aslan and Tanrıseven (2007).

Effect of Temperature on Enzyme Activity: The effect of temperature on enzyme activity was found by conducting the activity assay with 1 % (w/v) lactose solutions (pH was 4.5 and 5.5 for the 200 µL free or 0.317 g immobilized AOG respectively) at different temperatures according to the method described by Aslan and Tanrıseven (2007).

pH Stability: 200 µL free or 0.317 g immobilized AOG were incubated in sodium phosphate buffer solutions at various pH (3.0–8.0) at room temperature for 60 minutes and the retained activities were determined under standard assay conditions (Aslan and Tanrıseven, 2007).

Thermal Stability: 200 µL free or 0.317 g immobilized AOG were incubated in sodium phosphate buffer solutions (25 mM, pH 4.5 for free and 5.5 for immobilized enzymes) at temperatures from 30 to 80 ºC for 60 minutes and then the retained activities were determined under the standard assay conditions using the method described by Aslan and Tanrıseven (2007).

Kinetic Constants: Initial velocities for kinetic parameters were determined by performing the reactions between 200 µL free or 0.317 g immobilized AOG and lactose solutions at different concentrations (10 to 200 g/L) for 5 min. K_m and V_{max} were calculated from Lineweaver–Burk plots.

Operational and Storage Stabilities of the Immobilized AOG: Operational and storage stabilities of the immobilized AOG were determined by using the standard activity assay method, in repeated twenty batch experiments and every two days for fifteen days, respectively.

Hydrolysis of Milk Lactose by Using Immobilized AOG: 0.317 g immobilized AOG was reacted with 5 mL of semi-skimmed cow's milk at 55 ºC for 7 h. During hydrolysis, D-glucose content was determined with 30 min intervals by using the protocol of Wang (2018) based on the DNS method (Miller, 1959).

RESULTS AND DISCUSSION

Protein Assay: The amounts of proteins in the immobilization solution were calculated using Equation 3.

$$Y = 0.29958X \quad (3)$$

Accordingly, the enzyme concentration in a 5,2 mL immobilization solution containing 200 µL of free AOG was calculated to be 9.58 mg / mL. The enzyme concentration in the preparation of the powder AOG was also calculated as 958 mg / g by multiplying the amount of protein in the immobilization solution by the dilution factor (26). The calculated protein content of liquid AOG preparation is consistent with the declaration (β-Galactosidase content of powder enzyme preparation is between 80 – 97%) of the manufacturer company (Murphy, 2017).

Determination of β-Galactosidase Activity: The amount of D-glucose formed at the end of the reaction of 200 µL of free AOG at optimum activity assay conditions, was found to be 7965.3 µg by using Equation 4. The β-Galactosidase activity in 200 µL AOG solution was calculated using Equation 5. Accordingly, free AOG activity was determined to be 0.077 IU / mL. The β-Galactosidase activity of powder AOG preparation was also calculated to be 7.7 IU/g for lactose as substrate. The amount of free AOG having 1 IU activity was also calculated as 124.4 mg.

$$Y = 0.00104X \quad (4)$$

$$IU / mL\ Enzyme = \frac{Released\ D\text{-}Glucose\ (\mu mol)}{Enzyme\ used\ (mg)\ x\ Duration\ of\ reaction\ (min)} \quad (5)$$

Optimization of the Immobilization Procedure

Effect of Immobilization Buffer pH on Immobilization Efficiency: Table 1 shows the influence of pH on immobilization. Despite the lowest immobilization yield (79.34%) highest activity yield (92.16%) was achieved at optimum pH (4.5). The epoxy groups on Eupergit C can react with various reactive groups of enzymes in a wide pH range (0–12). But, the highest activity yield is usually attained at the optimum pH range in the immobilization

of enzymes with Eupergit C (Katchalski-Katzir and Kraemer, 2000).

Effect of Immobilization Buffer Molarity on Immobilization Efficiency: Despite the lowest immobilization yield (79.34%) highest activity yield (92.16%) was obtained for sodium phosphate buffer at 0.5 M (Table 2). On the other hand, when the molarity of immobilization buffer increased, activity yield was decreased while immobilization yield increased. Immobilization and activity yields by covalent enzyme immobilization on synthetic carriers such as epoxy carriers were often affected by the properties of the salts and their concentration (Smalla *et al*., 1988). The higher salt concentrations can change the three dimensional structures of enzyme molecules. Therefore, probably the increased buffer concentration has reduced the immobilization yield and activity yield.

Effect of Eupergit CM Amount on Immobilization Efficiency: Different amounts of Eupergit CM (100–600 mg) for 200 µL AOG were tested. Table 3 shows that the activity yield was decreased while the immobilization yield was increased when the amount of Eupergit CM was increased. Usage of higher amounts of Eupergit CM has resulted in the low activity yield. This is possibly the result of the deterioration of the three dimensional structure of enzyme molecules upon multipoint attachment of enzyme molecules to the support. Furthermore the reaction of epoxy groups in support with amino acid residues associated with the active site of enzyme molecules can lead to decreasing the activity.

Effect of Immobilization Duration on Immobilization Efficiency: As seen in Table 4, both of immobilization yield and activity yield are increased when the duration of immobilization increased until 96 hours. But, at the end of 120 hours, activity yield was decreased from 129.82% to 112.56%. The decrease in the activity yield after 96 hours is probably resulted from the destruction of enzyme active conformation due to the multipoint attachment of enzyme molecules to the supports. (Zhang *et al*., 2013). Consequently, by optimizing the immobilization conditions, 100% immobilization yield and 129.8% activity yield was achieved. Similar results are frequently encountered in the literature (Torres-Bacete *et al*., 2000; Aslan and Tanrıseven, 2007; Zarcula *et al*., 2009; Aslan *et al*., 2013; Aslan *et al*., 2014).

Characterization of Immobilized Enzyme

Optimum pH: According to Fig 1, the optimum pH of AOG was shifted from 4.5 to 5.5 by immobilization. The optimum pH of free and immobilized AOG was found as 4.5 (Husain *et al*., 2011). There are a lot of studies in the literature that report the changed optimum pH after immobilization. For example, the optimum pH of AOG had shifted from 4.0 to 4.5 and 5.0 after immobilization with different membranes (Mohy Elden *et al*., 1999). In another study, after immobilization with nylon Hydrolon membrane, the optimum pH of AOG had shifted from 4.0 to 4.5 (El-Masry *et al*., 2000). The optimum pH of the enzyme may shift to higher or lower levels around the optimum pH depending on the hydrophilic or hydrophobic interactions between the immobilization supports and the enzyme molecules. This phenomenon is known partitioning effect (Ferreira *et al*., 2003). On the other hand, immobilized AOG is more active than free AOG in the pH ranges tested. Similar results are often seen in other enzyme immobilization studies in the literature.

Optimum Temperature: Fig 2 shows that the optimum temperature range (55-60 °C) was not affected by immobilization. This result agree with previous studies. For example, Husain *et al*. (2011) determined the optimum temperature 50-60 ºC for AOG. It is also clear that the immobilized enzyme exhibits higher activity than the free enzyme at the entire temperature range tested. We see in Fig 2 that the optimum temperature of AOG wasn't changed after immobilization and the immobilized enzyme exhibit more activity than free enzyme at the tested temperatures range. Since immobilization increases the thermal stability of the enzyme, immobilized enzymes exhibit higher activity at high temperatures than free enzymes. Further, the optimum temperature may not changed.

pH Stability: As shown In Fig 3, the stability of immobilized enzyme is higher than of free enzyme. Furthermore, the immobilized enzyme is more stable than the free enzyme at pH values all tested. Since most of the functional groups on side chains of amino acids available on the surface of proteins involved to the formation of covalent bonds, changes at the pH of can not affect the three dimensional structure of enzyme molecules. Thus, immobilized enzymes can be more stable at extreme pH ranges.

Thermal Stability: Fig 4 shows the effect of temperature on the stability of the free and immobilized enzyme. It is seen that immobilized enzyme more stable than the free enzyme at the higher temperatures than 55 ºC. Covalent bonds between enzyme molecules and support generally strength the three-dimensional structure of the enzyme. Thus immobilized enzymes become more stable at high temperatures.

Kinetic Constants: The Michaelis-Menten (K_m) and maximum velocity (V_{max}) constants of the free and immobilized enzyme were calculated using the Lineweaver-Burk Plot (Fig 5). V_{max} value was increased from 0.366 g D-Glucose / L.min to 0.415 g D-Glucose / L.min, while K_m value was decreased from 307.7 g / L to 234.2 g / L. Km value indicates the affinity of an enzyme to a substrate. When K_m decreased, the affinity of the

enzyme to substrat is increased. Therefore, the higher activity of immobilized enzyme obtained can be explained with its lower K_m value.

Operational and Storage Stabilities of the Immobilized AOG: The activity of immobilized enzyme decreased to 99.3 % of initial value during the repeated twenty uses under optimum conditions. The activity of immobilized enzyme decreased to 98.3 % of initial value after fifteen days under optimum storage conditions. The immobilized AOG obtained in this study has higher operational and storage stabilities than previous studies. For example, native ZnO and ZnO-NP adsorbed β-galactosidase retained 61% and 75 % of the initial activity after seventh repeated use (Husain et. al., 2011). In another study, The entrapped crosslinked concanavalin α– β- galactosidase complex retained 95 % activity after seventh repeated use (Haider and Husain, 2007).

Hydrolysis of Milk Proteins by Using Immobilized AOG: Changes in the D-glucose concentration in the UHT cow's milk during hydrolysis were evaluated by using the graph of D-Glucose concentration against the time course (Fig 6). As seen in the figure, all of the milk lactose was completely hydrolyzed in four hours.

Conclusions: In this study, firstly AOG has been immobilized onto Eupergit CM with activity yield 129.82% by optimizing the immobilization conditions. The activity yield achieved in the presented study is higher than previous studies in the literature related to immobilization of AOG. As a second, the attained immobilized AOG has shown high operational and storage stability. Finally, the cow's milk lactose has been completely hydrolyzed by using immobilized AOG. As a consequent, the immobilized AOG achieved in this study can be used in the industrial production of lactose-free cow's milk.

Table 1. Effect of Immobilization Buffer's pH on Immobilization Efficacy

Immobilization Buffer pH	Immobilization Yield* (%)	Activity Yield **(%)
4.0	97.43	78.79
5.0	96.92	86.32
5.5	79.34	92.16
6.0	97.84	79.41
7.0	98.10	73.51

* 200 µL AOG were reacted with 100 mg of Eupergit CM in 5mL of phosphate buffers (0.5 M) at different pHs and room temperature with shaking at 150 rpm in an incubator for 16 hours.

** Free (200 µL) and 0.317 g immobilized AOG were reacted with 5 mL of 1 % (w/v) maltodextrin solutions (pH 4.5) at 150 rpm and at 55 ºC in an incubator for 60 minutes.

Table 2. Effect of Immobilization Buffer's Molarity on Immobilization Efficacy

Buffer Molarity (M)	Immobilization Yield* (%)	Activity Yield** (%)
0.5	79.34	92.16
1.0	90.54	77.59
1.5	92.70	66.73
2.0	94.17	53.42

* 200 uL AOG were reacted with 100 mg of Eupergit CM in 5 mL of different concentrated phosphate buffers (pH 4.5) at room temperature by shaking in an incubator at 150 rpm for 16 hours.

** Free (200 µL) and 0.317 g immobilized AOG were reacted with 5 mL of 1 % (w/v) maltodextrin solutions (pH 4.5) at 150 rpm and at 55 ºC in an incubator for 60 minutes.

Table 3. Effect of Eupergit CM Amount on Immobilization Efficacy

Eupergit CM (mg)	Immobilization Yield* (%)	Activity Yield** (%)
100	79.34	92.16
200	87.88	80.73
300	89.96	73.95
400	97.03	64.22

* 200 µL AOG were reacted with different amounts of Eupergit CM in 5mL of phosphate buffers (0.5 M, pH 4.5) and room temperature with shaking at150 rpm in an incubator for 16 hours.

** Free (200 µL) and 0.317 g immobilized AOG were reacted with 5 mL of 1 % (w/v) maltodextrin solutions (pH 4.5) at 150 rpm and at 55 ºC in an incubator for 60 minutes.

Table 4. Effect of Immobilization Duration on Immobilization Efficacy

Duration of Immobilization (hours)	Immobilization Yield* (%)	Activity Yield** (%)
24	79.34	92.16
48	84.02	104.52
72	93.39	118.02
96	100.00	129.82
120	100.00	112.56

* 200 uL AOG were reacted with 100 mg of Eupergit C in 5 mL of phosphate buffers (0.5 M, pH 4.5) at room temperature with shaking in an incubator at 150 rpm for different durations.

** Free (200 µL) and 0.317 g immobilized AOG were reacted with 5 mL of 1 % (w/v) maltodextrin solutions (pH 4.5) at 150 rpm and at 55 ºC in an incubator for 60 minutes.

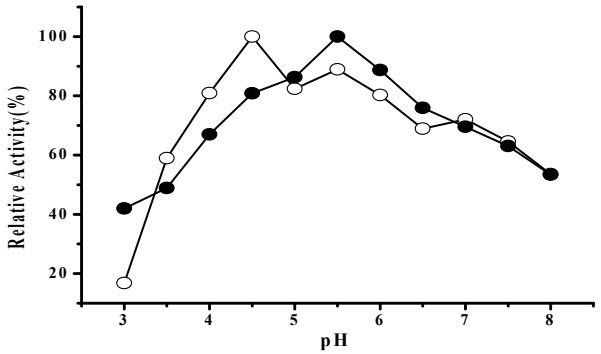

Fig 1. Optimum pH of free (○) and immobilized (●) AOG. The effect of pH on enzyme activity was investigated by performing the activity assay for 200 µL free and 0.317 g immobilized AOG with 1 % (w/v) lactose solutions, at different pH and at 55 ºC.

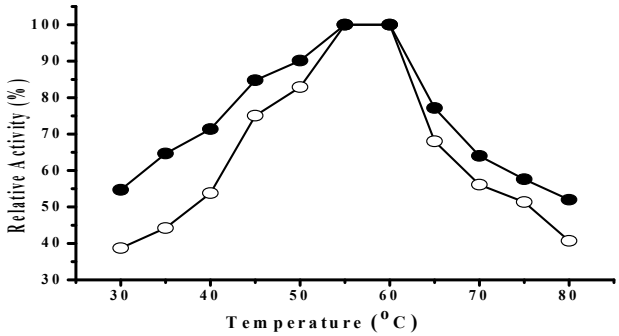

Fig 2. Optimum temperature of free (○) and immobilized (●) AOG. The effect of temperature on enzyme activity was found by conducting the activity assay for 200 µL free and 0.317 g immobilized AOG with 1 % (w/v) lactose solutions (pH 4.5 for free 5.5 for immobilized AOG) at different temperatures.

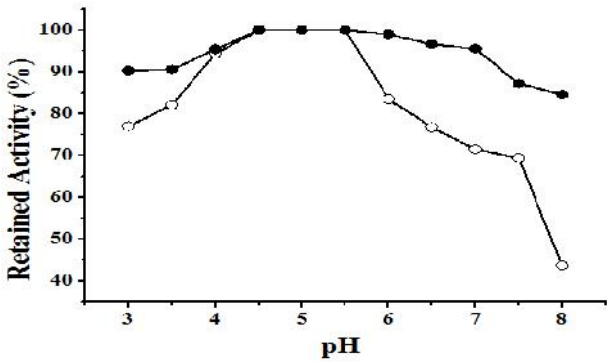

Fig 3. pH stability of free (○) and immobilized (●) AOG. 200 µL free and 0.317 g immobilized AOG was incubated in buffer solutions at various pH (3.0–8.0) and at room temperature for 1 h and the remaining activity was determined under standart assay conditions.

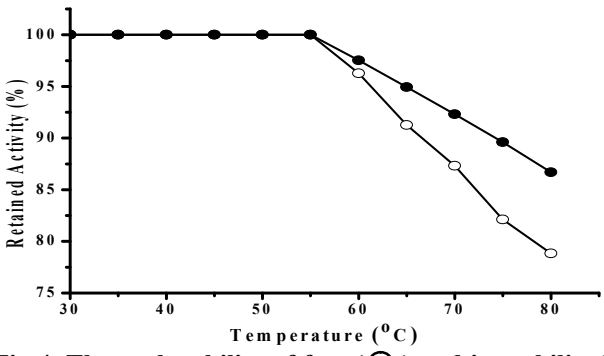

Fig 4. Thermal stability of free (○) and immobilized (●) AOG. 200 µL free and 0.317 g immobilized AOG was incubated in buffer solutions (25 mM, pH 4.5 for free and 5.5 for immobilized AOG) at temperatures from 30 to 80 ºC for 1 h and then the remaining activity was determined using the standart assay method.

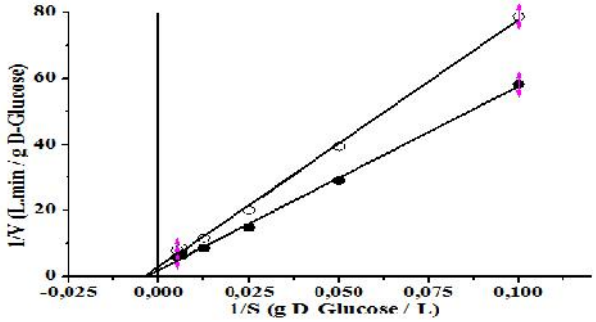

Fig 5. Lineweaver–Burk plots of free (○) and immobilized (●) AOG. Initial velocities for kinetic parameters were determined by performing the reactions between 200 µL free or 0.317 g immobilized AOG and the lactose solutions at concentrations (10 to 80 g/L) for 5 min.

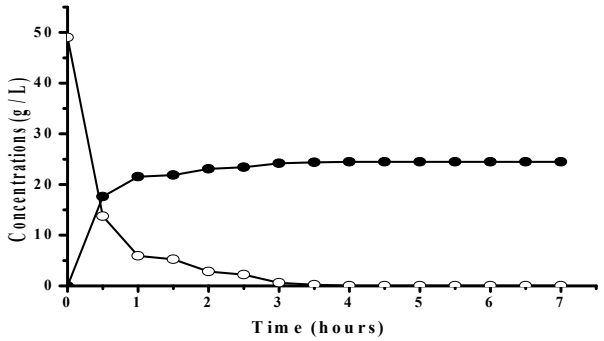

Fig 6. Hydrolysis of milk lactose by using immobilized AOG. 0317 g immobilized AOG was reacted with 5 mL of UHT cow's milk at 55 ºC for 7 hours and Lactose and glucose contents were determined with 30 min intervals using UV spectrophotometer. Lactose (○), glucose (●).

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