DOI : <http://doi.org/10.22438/jeb/40/5/PRN-118>

Optimization and immobilization of urease enzyme isolated from *Proteus mirabilis* on alginate beads

Paper received: 14.01.2016

Revised received: 28.02.2019

Re-revised received: 28.04.2019

Accepted: 29.04.2019

Authors Info

A.T. Tetiker^{1*} and F. Ertan²¹Vocational College of Health Services, İstanbul Rumeli University, İstanbul, 34570, Turkey²Department of Biology, Faculty of Science, Trakya University, Edirne, 22030, Turkey*Corresponding Author Email : aylinturksever@hotmail.com

Edited by

Dr. Sumati Gaumat

Reviewed by

Dr. Brijesh Kumar Mishra
Dr. R. B. Raizada

Abstract

Urease enzyme was isolated from *Proteus mirabilis* and immobilized in alginate beads. Various parameters such as optimum pH, optimum temperature, pH stability, thermal stability, reusability, storage stability and substrate concentration were investigated and the findings were compared with free urease enzyme. Immobilization yield was calculated as 85 %. Optimum temperature was found to be 40°C for free and immobilized urease. Thermal stability of the immobilized urease enzyme was significantly better than the free enzyme. Optimum pH for free and immobilized urease was be 7.0. Immobilized and free urease enzymes protected their stability at pH 7.0 and 8.0 in a similar way. Immobilized enzyme maintained 55% of their initial activity after 12 repeated use of enzyme. It was found that storage stability of immobilized enzyme was better than that of free enzyme. K_m and V_{max} values from the Lineweaver-Burk plots were calculated.

Isolation of urease enzyme from *Proteus mirabilis* and its immobilization on alginate beads.

Estimation of some parameters like thermal stability, temperature, optimum pH, reuse and storage ability and their comparison with free enzyme.

Entrapped urease on alginate beads showed 85% efficiency and showed better performance than free urease enzyme.

Urease isolated from *Proteus mirabilis* can be used as an important resource for biotechnological and industrial applications.**Key words:** Alginate, Immobilization, *Proteus mirabilis*, Urease

How to cite : Tetiker, A.T. and F. Ertan: Optimization and immobilization of urease enzyme isolated from *Proteus mirabilis* on alginate beads. *J. Environ. Biol.*, **40**, 1123-1128 (2019). DOI : <http://doi.org/10.22438/jeb/40/5/PRN-118>

Introduction

Urease (urea aminohydrolase; EC 3.5.1.5.) is a nickel dependent metalloenzyme which hydrolyzes urea into ammonia and carbon-dioxide (Follmer *et al.*, 2004). The enzyme urease is found in plants, bacteria and fungi (Banchmeier *et al.*, 2002). The most significant task of urease is to enable the organism to utilize urea as a source of nitrogen. Urease plays an important role in pathogenesis of ailments in positive bacteria, fungi, human beings and animals (Mobley *et al.*, 1995). Urease positive microorganisms constitute ammonia and carbon-dioxide by hydrolyzing urea in the environment so that they provide a living space for themselves by changing the pH of environment into alkaline direction. In a research, it has been revealed that urease is an important virulence factor in urinary infection (Konieczna *et al.*, 2012). The interest in urease enzyme in biotechnological studies has been gradually increasing. Urease has several industrial practices such as in estimation level of urea in biological liquids, as a diagnostic kit, as a biosensor in blood urea detection, detracting urea from blood through artificial kidney, detracting urea from fruit juice and food and beverages containing alcohol in food industry, removal of urea from waste water is one of the most important reasons to be a limelight for researchers (Dindar *et al.*, 2011; Geweely, 2006).

In order to solve certain problems such as reusability, cost, contamination, scarcity in the amount coming into being in the utilization of free enzyme in industrial practices, immobilized enzyme is used (Franssen *et al.*, 2013). Immobilization of enzyme is one of important opportunities of biotechnology. The immobilized enzyme provides as an advantage by reducing the production costs thanks to their reusability resulting from their specifications, protection their catalytically activities even at high temperature and pH and easy decomposition in reaction environment. There are several methods for immobilization, of enzymes among which calcium alginate gel has many advantages owing to its features such as biocompatibility to immobilization method, being non-toxic, not giving any harm to enzyme by realizing the immobilization under moderate circumstances and being a cheap method (Yagar *et al.*, 2008).

Alginate has been used in immobilization of various enzymes such as inulinase (Catana *et al.*, 2005), amylase (Konsoula *et al.*, 2006; Ertan *et al.*, 2007) xylanase (Kumar *et al.*, 2017), pectinase (Olivera *et al.*, 2018), polygalacturonase (Carvalho Silva *et al.*, 2018). Previous studies, have reported immobilization of urease into various support materials such as chitosan (Kumar *et al.*, 2009; Bankapalli *et al.*, 2011), chitosan and poly(acrylamide-co acrylic acid/kappa carrageenan (Kara *et al.*, 2006), ZnO nanoparticle (Eghbali *et al.*, 2015), pyrrole-3-carboxylic acid (Karazehir *et al.*, 2016), magnetic support (Vargas *et al.*, 2017), cellulose (Miaomiao *et al.*, 2018), silica gel (inorganic SiO) (Mondal *et al.*, 2019). In this study, urease enzyme isolated from *Proteus mirabilis* was immobilized into alginate gel beads. The operating conditions of immobilized and free enzyme were optimized and compared with each other. Within this purpose, the specifications of enzymes such as

temperature, pH, substrate concentration, pH stability, thermal stability, reusability and storage stability were also studied.

Materials and Methods

In this study, *Proteus mirabilis* was procured from Central Laboratory of Trakya University, Faculty of Medicine. Urease was isolated from *Proteus mirabilis*, a common cause of urinary tract infections in individuals with functional or structural abnormalities or with long-term catheterization, forms bladder and kidney stones as a consequence of urease-mediated urea hydrolysis. Sodium alginate was purchased from Fluka Biochemika CaCl₂ from Sigma and urea from Merck, respectively. The other chemicals used in the experiment were of analytical grade.

Proteus mirabilis was produced at 37°C, in 24 hour shaken water bath; in liquid urease production medium which consisted 10 g glucose; 5 g peptone; 2.5 g yeast extract; 1 g KH₂PO₄; 1 g K₂HPO₄; 2.5 g NaCl; 2.5 g urea; 0.025 g MgSO₄; 0.025 g NiSO₄ and 500 ml distil water. Supernatant liquid was added by centrifuging the medium for 15 min at 12000 rpm. Precipitate was washed with distil water and re-centrifuged. Over the precipitate, buffer of 2.5 ml Tris-HCl pH 7.0 was added and mixed with a glass stirrer; this mixture was concentrated in an Erlenmeyer, and the cell wall of bacteria was decomposed through sonication with a sonicator (Bandelin UW 2200/60 Hz) for 1 minute 5 times. Lastly, the process of centrifuge was conducted and supernatant has been used as a crude enzyme source.

Urease activity of both free and immobilized enzyme were assessed by Nessler ammonia method (Golterman, 1991). In this study, urea solution (1% in 50mM Tris HCl buffer, pH 7.0) was used as substrate. Reaction mixture was prepared by adding 500 ml of free enzyme or 0.5 g immobilized enzyme, 2.5 ml urea and 2.5 ml Tris-HCl buffer. The mixture were incubated at 50°C for 40 min. Then, 100 ml Nessler reagent was added to the mixture. The absorbance was measured at 425nm. The slope of the ammonium calibration curve was used in order to calculate enzyme activity. One unit of enzyme is defined as the amount of enzyme which hydrolyses 1 micromol of ammonia in 1 min. All the were performed at least 3 replicates and expressed as. Mixture of 1:1 volume of sodium alginate solution and urease enzyme solution were prepared to give a 3% final concentration of sodium alginate solution in mixture. The mixture obtained was extruded drop by drop through a syringe into a gently stirred 3% CaCl₂ solution. It was kept at 4°C for 50 min in buffer solution until maturation. Enzyme containing calcium alginate beads were separated from CaCl₂ solution by filtration. The mature beads were washed in cold Tris-HCl buffer (50 mM, pH 7.0). Bradford method was used for determining protein content free and immobilized enzymes. (Bradford, 1976). Immobilization efficiency was calculated as follows:

$$\text{Immobilization efficiency (\%)} = a_{\text{imm}} / a_{\text{free}} \times 100$$

a_{imm} = specific activity of immobilized enzyme (U mg⁻¹ protein)

a_{free} = specific activity of free enzyme (U mg⁻¹ protein)

The pH range of 6.0-10.0 was used for the investigation of the effect of pH on the activity of the free and immobilized urease. The pH stability of the free and immobilized urease was determined by incubating in substrate free different buffers (Tris-HCl buffer: pH 6.0-8.0 and borate buffer: pH 9.0-10.0) for 30 min at 4°C. The activity and pH stability were determined at the en of this period.

The effect of temperature on free and immobilized urea activity was detected within the measurements after incubation of reaction mixture at varying temperatures (35, 40 and 45°C). Thermal stability of both enzyme forms were investigated by incubating the enzyme samples at temperature ranging between 35-45°C and at different durations (10, 30 and 60 min) without substrate in Tris-HCl buffer at pH 7.

Varying concentrations (0.08, 0.16, 0.24, 0.32, 0.40, 0.48, 0.56, 0.64, 0.72, 0.82 mmol) of urea precipitate was prepared to

determine the effect of substrate on enzyme activity. The K_m and V_{max} values of immobilized and free urea enzymes were calculated from Lineweaver-Burk plots.

Enzyme activity of immobilized urease on alginate beads was measured several times successively so as to determine the reusability of *Proteus mirabilis* urease immobilized into alginate gel. Following each measurement, the beads were washed with distil water and the activity detection was re-performed.

The activities of free and immobilized urease at 4°C were estimated and compared.

Statistical treatment of immobilization conditions and reaction optimization was carried out by multivariate analysis. Results were evaluated using the software STATISTICA 7.0 (Statsoft Inc. 2325 East 3rd Street, Tulsa, OK 74104, USA), and the model was simplified by dropping terms that were not regarded

Fig. 1: Effect of pH on free and immobilized urease activity.

Fig. 2: Effect of pH stability on free and immobilized urease activity.

Fig. 3 : Effect of temperature on free and immobilized urease activity.

Fig. 4 : Effect of temperature on stability of free urease.

as statistically significant ($p > 0.05$) by the analysis of variance (ANOVA). Data of urease were processed by central mean and standard deviation measurements and by the Tukey test to determine significant differences among the means. All tests were conducted in triplicate and the level of significance was 99 %)

Results and Discussion

Immobilized enzymes have attracted great attention in the last couple of years due to the feature of reusability opportunity and their protection the stability of high-priced enzymes. The entrapment technique being one of the immobilization techniques may be defined as locking the enzyme physically in a space or network. In urease immobilization, various natural and synthetic materials have been used as conveyor. Urease has been used within technical and therapeutic purposes by entrapping to tube membranes in recent years. The entrapping studies of sodium alginate and carrageenan being

natural polymers have increased, as well (Baysal and Karagöz, 2005; Mahajan *et al.*, 2010). Owing to immobilization on alginate gel being a fast and easily practicable method, and alginate having a pore structure enabling substrate access and at the same time restricting enzyme break, being non-toxic and biocompatibility, it is preferred in biotechnological and industrial studies. In this study, urease enzyme isolated from bacteria, *Proteus mirabilis* was immobilized on alginate gel.

pH is one of important parameters affecting enzyme activity. In the studies having been conducted before the optimum pH value of urease enzyme has been revealed to be changing between 4.5 and 9 (Das *et al.*, 2002; Tai and Mobley, 1993; Fidaleo and Lavecchia, 2003). In this study, optimum pH of *Proteus mirabilis* urease for both forms has been found to be 7.0. There has been no remarkable change over pH profile by immobilizing urease (Fig. 1). In the study of pH stability immobilized and free urease has protected their stability similarly

Fig. 5: Effect of temperature on stability of immobilized urease.

Fig. 6: Effect of substrate concentration on soluble and immobilized urease activity.

Fig. 7: Repeated use of immobilized enzyme.

Fig. 8: Storage stability.

at pH 7.0 and 8.0 (Fig. 2). The optimum temperature of urease enzyme changes according the enzyme resource being studied (Mobley *et al.*, 1988; Pervin *et al.*, 2013; Krajewska *et al.*, 2012). In our study, the optimum temperature value of free and immobilized urease has been determined to be 40°C (Fig. 3).

Immobilization protect the tertiary structure of enzymes from environmental factors thereby increasing the thermal stability of immobilized enzyme (Kumar, 2012). Generally, the activity of immobilized enzyme is stated to be more resistant when compared with free enzyme in terms of temperature and denaturing agents (Danial *et al.*, 2015; Gouda *et al.*, 2002; Sari *et al.*, 2012). In this study, the optimum temperature of free and immobilized urease was determined to be 40°C. When both enzyme forms were kept at 30 and 35°C for 60 min, it was observed that they maintained their initial activities. Immobilized enzyme had maintained 88% of its initial activity when kept at 40°C for 60 min, whereas free enzyme had maintained only 75% of initial activity. When kept at same temperature for 60 min, free enzyme lost approximately 58% of its initial activity. However, the immobilized enzyme has been observed to lose only 29 % of its initial activity (Fig.4, Fig. 5). Previous studies, enzyme activities were shown to be kept more stable in immobilized enzymes than free enzymes (Ertan *et al.*, 2007).

The K_m values of free and immobilized enzymes in the present study were calculated according to Lineweaver-Burk plot. It was found to be 2 mM for free enzyme, while for immobilized enzyme it was 0.4 mM. The V_{max} value of immobilized and free enzyme was found to be 35 U ml⁻¹ and 40 U ml⁻¹, respectively (Fig. 6). Usage of enzymes in free form over and over is not feasible, therefore enzymes used for industrial purposes are immobilized repeatedly (Khan and Alzohairy, 2010; Singh *et al.*, 2013). In this study, immobilized urease was used 12 times actively over and over (Fig.7). It has been stated that free enzyme loses its activity considerably during storage in immobilization studies (Pithawala, 2010). In this study, the immobilized enzyme protected its initial activity for 4 days. The free enzyme had lost approximately 20 % of its activity on 4th day. It was observed that free enzyme had lost almost all its activity on 11th day whereas immobilized enzyme protected 53 % of its activity (Fig. 8). Enzyme immobilization was carried out to increase the reusability and stability of enzymes in extreme conditions but it is also advantageous for storage conditions. Although in literature, urease from *P. mirabilis* was not immobilized, the immobilization of urease enzyme in this study elevated its storage potential.

In conclusion, urease enzyme isolated from *Proteus mirabilis* immobilized on alginate beads has advantage over free enzyme in terms of thermal stability, storage stability and reusability. Besides, alginate being cheap with a simple structure has advantages compared with other methods. *Proteus mirabilis* urease has been entrapped on alginate beads for the first time in this study and immobilization efficiency of urease was 85%. Urease enzyme isolated from *Proteus mirabilis* can be an important source in biotechnological and industrial applications.

Acknowledgment

This research was supported by Trakya University Research Foundation (Project Number: TUBAP-2013-147).

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