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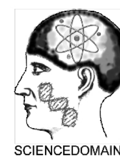

Characteristics of Penicillin G Acylase Immobilized onto Iron Oxide Nanoparticles

Momen Seyed Bahman[1], Atyabi Seyed Mohammad[1], Azim Akbarzadeh[1], Salimi Mona[2], Sedigeh Hatami Gigloo[1], Farhangi Ali[1] and Norouzian Dariush[1*]

[1]Pilot Biotechnology Department, Pasteur Institute of Iran, Tehran 13164, Iran.
[2]Physiology and Pharmacology Department, Pasteur Institute of Iran, Tehran 13164, Iran.

Authors' contributions

This work was carried out in collaboration between all authors. All authors have read and approved the manuscript.

Research Article

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ABSTRACT

Penicillin G acylase was immobilized onto iron oxide nanoparticles coated with polyethyleneimine and then cross linked with glutaraldehyde solution. The FTIR spectrum of immobilized enzyme showed peak at 1648cm^{-1} which can be attributed to the C=N bonds of Schiff's base linkage formed between glutaraldehyde and amino group of penicillin G acylase. By considering the FTIR spectrum of nano particle coated with polyethyleneimine, adsorption of penicillin G acylase has taken place and then glutaraldehyde cross linked enzyme onto activated support. Catalytic properties of nano penicillin G acylase were improved upon immobilization as compared to its free counterpart. The optimal pH and temperature were determined to be 7.0, 10.0, 50 and 75ºC for free and immobilized penicillin G acylase, respectively. Thermal stabilities of both nano and free penicillin G acylase were studied .The Km value of immobilized nanozyme was calculated from Lineweaver Burck plot to be 0.23 µM while that of free penicillin G acylase was 0.28µM. In this way nano penicillin G acylase with improved catalytic properties was developed as compared to its soluble counterpart.

Keywords: Immobilized; nanopenicillin G acylase; polyethyleneimine; kinetic properties.

**Corresponding author: Email: dnsa@pasteur.ac.ir;*

1. INTRODUCTION

Penicillin G acylase (penicillin amidohydrolase; E.C.3.5.1.11) is an enzyme produced by bacteria as well as fungi in order to acylate/deacylate penicillin G to its constituent 6-aminopenicilanic acid and phenyl acetic acid or vice versa which is termed as hydrolysis (H) and synthesis (S), (Fig. 1) respectively. In addition to hydrolytic or synthetic application of penicillin G acylases, they can be used in several other biotechnological applications, such as peptide synthesis and racemic resolution [1]. Due to hydrolytic and synthetic ability of penicillin G acylase and importance of semi synthetic antibiotics, the enzyme has profound uses in related industry. Penicillin G acylase is employed in industrial production of semi synthetic antibiotics which are mainly the most used antibiotics in treating the infectious diseases [2]. Thus Semi-synthetic β-lactam antibiotics are produced in several tons annually. It may be possible to produce new semi synthetic β-lactam antibiotic in future [3]. In industry, the stability of immobilized enzyme during production is the most important parameter in order to achieve good yields and to economize the process as well [4]. Therefore, penicillin acylase in its soluble form is unstable, cannot be separated from the reaction mixture easily thereby adding to the production cost of the final product thus such a commercially and industrially important enzyme has been immobilized through different techniques viz adsorption, ionic and covalent binding or entrapping it into lattice of the matrices using various supports by many investigators [5,6,7,8,9,10,11,12]. Each technique or method has its own limitation and disadvantages where immobilization method can alter kinetic properties of immobilized enzyme as compared to its soluble counterpart. The changes can be brought about by the supports or the reagents employed. Investigators are trying to employ technique and reagents which will improve the kinetic properties of the enzyme under immobilization process as compared to its soluble counterpart [13,14,15,16]. But attempts are made to stabilize and improve the enzymes properties by immobilization [17,18,19]. In recent years, different supports at down to nano scale have been fabricated in order to be explored in enzyme immobilization processes due to their large surface area [20]. To avoid diffusional limitation, the enzymes are to be immobilized on activated supports' surfaces [21]. In this article attempts are made to immobilize penicillin G acylase onto polyethyleneimine coated iron oxide nanoparticles so as to study the kinetics properties of obtained immobilized penicillin G acylase. The developed immobilized penicillin acylase with improved kinetic properties could be employed to catalyze hydrolytic/synthetic reactions in production of 6-aminopenicillanic acid/ semi synthetic antibiotics.

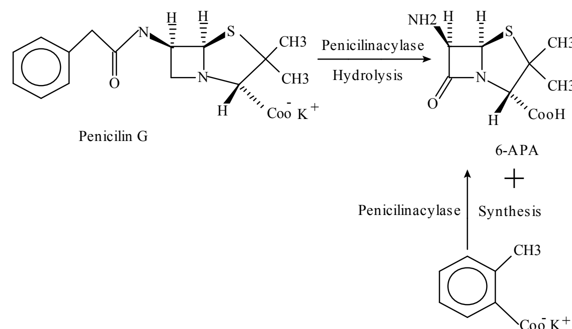

Fig. 1. Penicillin G acylase catalyzed reaction (S/H)

2. MATERIALS AND METHODS

2.1 Materials

Penicillin G acylase, 6-aminopenicillanic acid (6-APA), polyethylenimine (PEI), phenylmethylsulfonyl fluoride (PMSF), *p*-dimethylaminobenzaldehyde (*p*-DMBA) were obtained from Sigma, USA. Benzyl penicillin was procured from local market; other reagents used were of analytical grade.

2.2 Methods

2.2.1 Synthesis of iron nanoparticles

Super paramagnetite iron particles were prepared according to the method described by Kouassi et al. [22] Fig. 2.

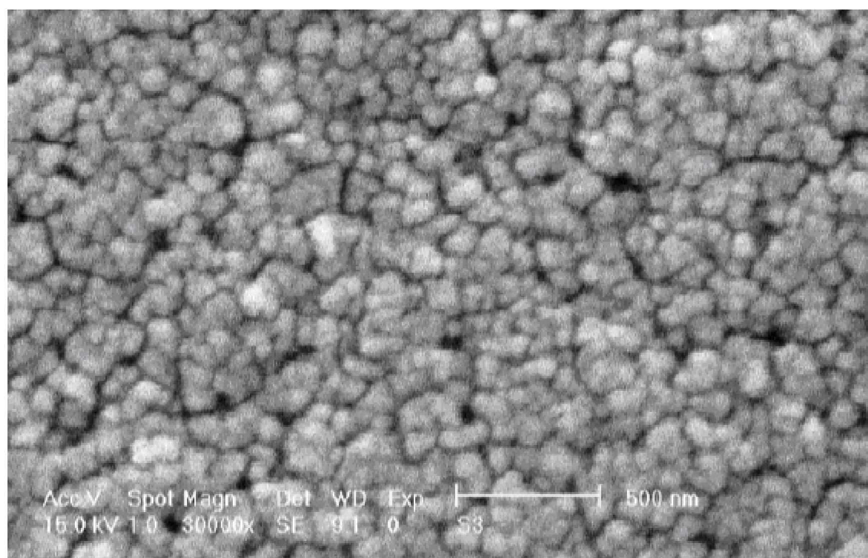

Fig. 2. SEM micrograph of synthesized superparamagnetite iron oxide nanoparticles

2.2.2 Penicillin G acylase activity

Penicillin acylase activity was determined as reported by Norouzian et al. [9] measuring the amount of 6-aminopenicillanic acid (6-APA) formed at 35ºC ±1ºC, employing 2% (w/v) benzyl penicillin prepared in 0.1 M phosphate buffer pH7.5.The formed 6-APA was estimated with *p*-dimethylaminobenzaldehyde (*p*-DMBA).One unit of enzyme activity was defined as the amount of enzyme catalyzing the hydrolysis of penicillin G to produce 1 µmol of 6-aminopenicillanic acid in 1 min under the above assay condition.

2.2.3 Immobilization process of penicillin G acylase by surface response methodology through central composite design

Penicillin G acylase was immobilized onto synthesized super paramagnetite iron oxide nanoparticles as reported by Atyabi et al. [23]. In brief, the factors could influence the immobilization process were considered to be; enzyme concentration (U/ml/min) weight of iron oxide nanoparticles (µg), concentration of polyethylenimine (% v/v), reaction temperature (ºC) and reaction time(h). The immobilization process was optimized by surface response methodology (SRM) through central composite design (CCD) employing Ease State program, version 6.

2.2.4 FTIR studies

Synthesized iron oxide nanoparticles were coated with polyethylenimine (PEI), penicillin G acylase was coupled to the nanoparticles and then cross linked with glutaraldehyde. This process in each step was subjected to Fourier Transmitter Infra Red spectroscopy.

2.2.5 Active site titration

Free and immobilized penicillin G acylases were titrated with different concentration of phenylmethylsulfonyl fluoride (PMSF) as described by Van Langen et al. [24].

2.2.6 Operational stability of nano penicillin G acylase

The immobilized enzyme was added into 2% of penicillin G solution, the hydrolysis reaction was carried out under agitation at 35ºC for 120 min, and the hydrolysis reaction was performed 6 times, i.e. the immobilized enzyme was used repeatedly six times with the duration of each cycle 2 hours. During the hydrolysis the pH was maintained at 7.5

3. RESULTS AND DISCUSSION

Immobilization process of penicillin G acylase was optimized by using surface response methodology as reported by Atyabi et al. [23].The factors involved in immobilization process were enzyme concentration(U/ml), weight of support(µg) , concentration of polyethylenimine (v/v), temperature of the reaction (ºC) and time of reaction (h) [25]. Penicillin G acylase attached to polyethylenimine coated iron oxide nanoparticles was further cross linked by 0.5%(v/v) glutaraldehyde solution. This methodology has been employed to optimize biotechnological processes [26,27,28,29,30]. Furthermore, polyethylenimine "a polycationic reagent imparts positive charge onto surface of inert support", was considered another factor to play a role in immobilization process. Polyethylenimine coated support can be employed to immobilize proteins ionically, retaining the enzyme on the support even at very high ionic strength. The ionic immobilization of enzymes on PEI activated supports will preserve almost enzymes' catalytic activity and, may impose rigidification of enzyme causing stabilization. This can be considered as a positive effect of enzyme immobilization on PEI coated supports [31]. There are reports showing PEI has been employed to functionalize the inert supports [32,33,34,35,36]. Furthermore, Figs. 3 and 4 show FTIR spectra of immobilized penicillin G acylase on nano iron oxide. It shows peaks at 1648cm^{-1} which can be attributed to the C=N bonds of Schiff's base linkage formed between glutaraldehyde and amino group of penicillin G acylase. The features at 2948 and 2855 cm^{-1} represent the asymmetric and symmetric stretching of the CH respectively ,whereas at 2730 cm^{-1} defines the asymmetric and symmetric stretching of aldehyde groups. The weak bonds at approximate 1721 cm^{-1}

was assigned to the stretching of CO groups of carboxylate and non conjugated aldehyde. A broad absorption between 3600 and 3200 cm^{-1} is associated with the stretching modes of hydrogen bonded hydroxyl group in nano iron oxide. By considering the FTIR spectrum of nano particle coated with polyethylenimine, adsorption of penicillin G acylase has taken place and then glutaraldehyde covalently cross links enzyme onto activated support. The latter reagent provides aldehyde group to PEI/nanoparticles thus enzyme links to the surface of the support through Schiff's base between amino group of penicillin G acylase and aldehyde group of the matrice. Free and immobilized penicillin G acylase were subjected to active site titration using specific active site probe namely phenylmethylsulfonyl fluoride(PMSF) which irreversibly binds to serine present at the active site of penicillin G acylase. Free and immobilized penicillin G acylase were totally inhibited by different concentration of phenylmethylsulfonyl fluoride indicating penicillin G acylase has been immobilized onto the activated support [24]. The kinetics properties of immobilized penicillin G acylase were further studied. The kinetic properties of free and nano-penicillin G acylase are summarized in Table 1. As it can be seen from the Fig. 5, there are shifts in pH of immobilized penicillin G acylase as compared to free enzyme which could be due to fairly large quantities of protonated amino group of polyethyleneimine activated nano iron oxide support so this feature makes the activated surface positively charged [16]. Fig. 6 shows increase in optimal temperature of immobilized enzyme [13,37,38]. This increase in optimum temperature would be advantageous in using this type of support to immobilize other enzymes such as glucose isomerase, glucoamylase, cellulase wherein the viscosity of the medium could be lowered at higher temperature [21]. The apparent K_m and V_{max} of free and immobilized enzyme was studied by Lineweaver-Burk plot starting from high concentration of penicillin G solution/suspension to the lowest possible concentration showing improvements in apparent affinity of the substrate towards the immobilized penicillin G acylase (Table 1).

Table 1. Summary of kinetic properties of penicillin G acylase in two states

State of penicillin acylase	Optimum pH	Optimum temperature	Apparent K_m	V_{max}
Free enzyme	7.0	50ºC	0.28 µm	8.3U /min/ ml
nanozyme	10	75ºC	0.23 µm	11.3 U/ min/100µg of support

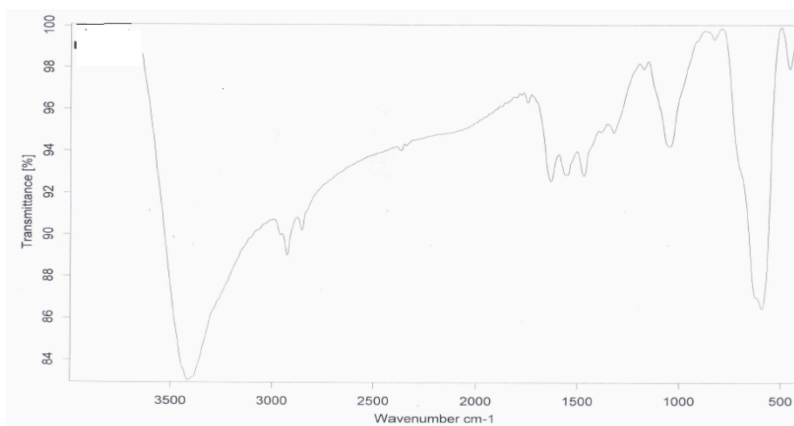

Fig. 3. FTRI of iron oxide nanoparticles coated with PEI

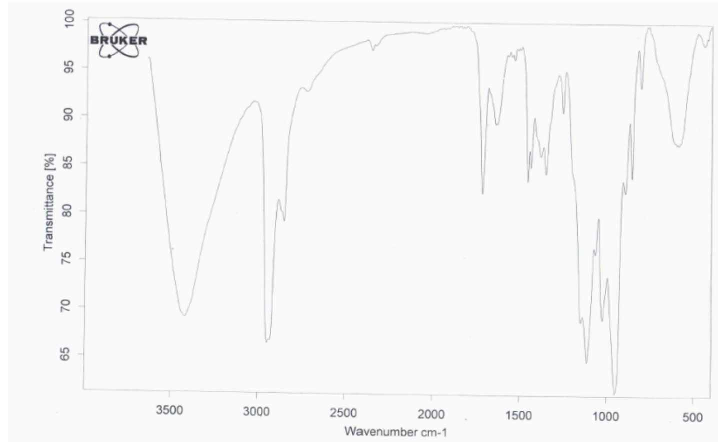

Fig. 4. FTRI of iron oxide nanoparticles coated with PEI+enzyme+glutaraldehyde

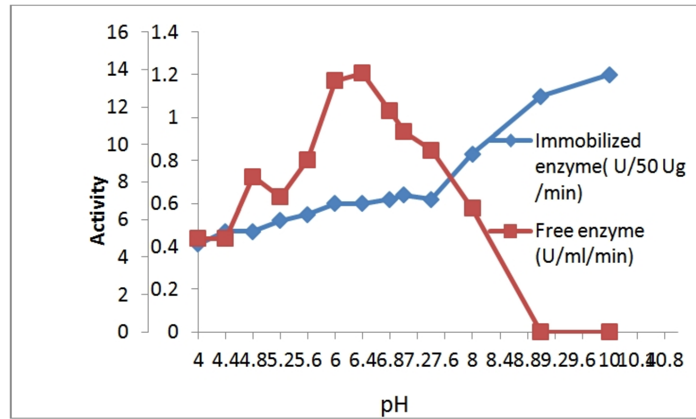

Fig. 5. Optima pH of immobilized and free penicillin G acylase

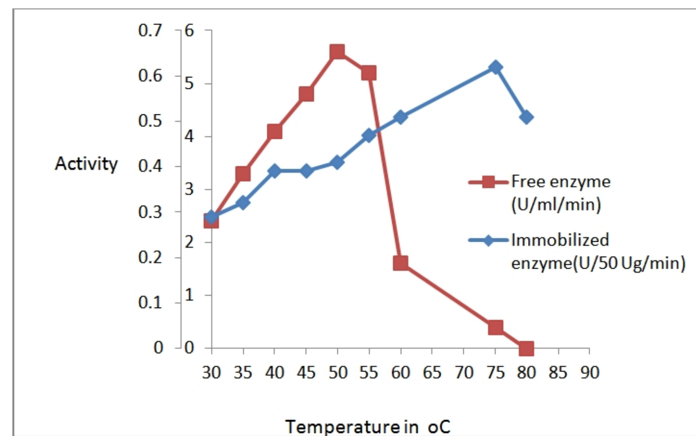

Fig. 6. Optima temperatures of immobilized and free penicillin G acylase

The operational stability of the immobilized enzyme was studied by hydrolysis reaction of penicillin G solution. The operational stability of nano penicillin G acylase in 6 continuous cycles maintained almost 90% of its original activity (Fig. 7) while Eldin et al. [39] studied glutaraldehyde activated NH_2 -PVC to immobilize penicillin G acylase. They showed that operational stability maintained only 40% of its original activity in 10.5 hours of working.

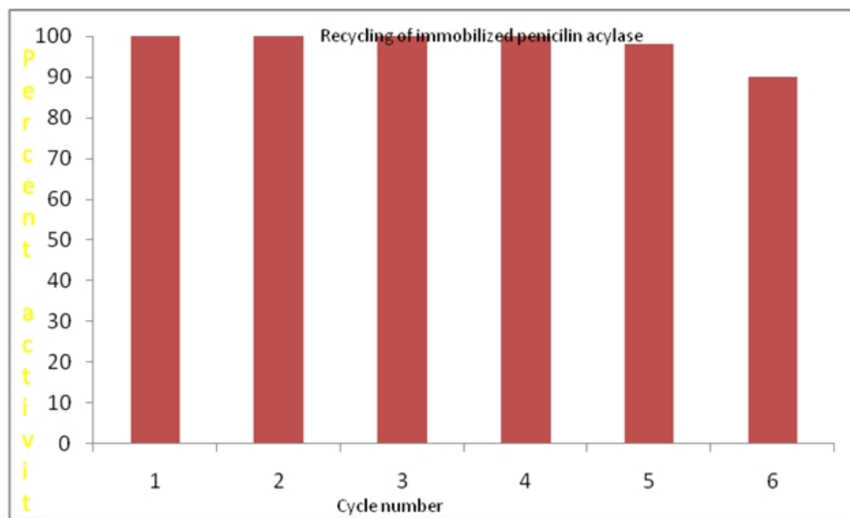

Fig. 7. Optima temperatures of immobilized and free penicillin G acylase

4. CONCLUSION

Penicillin G acylase is one of the industrially and commercially important enzyme used to produce semi synthetic antibiotic so as to treat the infectious diseases. Therefore to economize and ease the down stream processing attempts are made to develop an immobilized penicillin G acylase. Stabilization of the enzyme by immobilization employing inert supports at nano scale level could exploit the enzyme which is unstable. In this way, we could develop a nanozyme with improved catalytic properties.

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COMPETING INTEREST

The authors of this manuscript thereby declare existence of no competing interest.

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