ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Characteristics of immobilized bacterial D-hydantoinase on alginate

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Manuscript Info

Manuscript History:

Received: 17 March 2015
Final Accepted: 28 April 2015
Published Online: May 2015

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Abstract

D-hydantoinase (E.C.: 3.5.2.2) was isolated form *Bacillus theorgensis*. The enzyme activity was enhanced by Triton-X100 and Tween-20. ATP up to 8 mM activated the enzyme activity in concentration-dependent manner. The enzyme was inhibited by EDTA, *o*-phenanthroline, *α*-dipyridyl and hydroxyquinoline. EDTA was the weakest chelating agent. C_{50} for the other three compounds were 6.5, 4, and 3.3 mM. D-hydantoinase was activated by Cu^{2+}, Mn^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}, Co^{2+}, Ca^{2+} and Ni^{2+}. Ca^{2+} was the best activator. However, the enzyme was inhibited by Cu^{2+} and Fe^{2+}. The enzyme was immobilized on alginate beads. Increasing sodium alginate concentration up to 3% (w/v) resulted in continuous increase of immobilization yield. The optimal time of immobilization was 5h. Increasing of $CaCl_2$ concentration up to 3% (w/v) resulted in corresponding increase in the immobilization yield. Potassium phosphate buffer at 150 mM was better than Tris-HCl buffer for enzyme immobilization.

INTRODUCTION

Microbes serve as one of the largest and useful sources of many enzymes (Adrio and Demain, 2008). Advances in the field of molecular biology of microorganisms have opened up new horizons in the applications of new enzymes for developing novel products and applications (Barclay *et al.*, 2002).

The high specificity, fast action and biodegradability by enzymes allow enzyme-assisted processes in industry to run under milder reaction conditions, with reduction of waste generation and improving yields (Adrio and demain, 2014).

The importance of D-hydantoinase in the industrial bioconversion of amino acids lead to concentrate the work on its reaction mechanism, biochemical properties, and protein conformation have been extensively investigated (Xu *et al.*, 2003; RadhaKishan *et al.*, 2005).

Hydantoinase plays particular role in industry in the preparation of optically active compounds (May *et al.*, 2000; Scheomaker *et al.*, 2003). Combinations of hydantoinase with carbamoylase form biocatalysts for production of nonproteinogenic amino acids from racemic hydantoins (Altenbuchner *et al.*, 2001; Scheomaker *et al.*, 2003).

The free enzymes are unstable to fulfill economical requirements for an industrial purpose. Immobilized D-hydantoinase is used to produce D-amino acids (Chen *et al.*, 1999; Arcuri *et al*., 2002). The immobilized enzymes are used in industrial bioprocesses especially in nutritional, food and pharmaceutical technologies (Sheldon, 2007).

There are many reasons for using the enzyme in the immobilized form. First, the easy of handling of the enzyme, second its facile separation from the product, third it helps to prevent the contamination of the substrate with enzyme/protein or other compounds, which decreases purification costs (Spahn and Minteer, 2008), and forth reuse of costly enzymes, with longer half-lives and less degradation, (Shi *et al.*, 2011).

Enzyme immobilization has been implemented on a larger scale, in the food industry and in the manufacture of fine chemicals and pharmaceuticals (Krajewska, 2004).

The aim of the present investigation was to find out whether D-hydantoinase from *Bacillus theorgensis* is particulate enzyme and to immobilize it on alginate beads for investigating its characteristics as immobilized enzyme.

Materials and Methods

Growth medium

The growth medium of *B. theorgensis* contained 10 g/l sucrose, 10 ml corn steep liquor, 0.1 g yeast extract, 3 g NaCl, 2 g KH_2PO_4, and 0.25 g $MgSO_4$ at pH 8.0. The effects were studied separately using D-hydantoin as substrate and testing for production of *N*-carbamoyl-glycine. All media were sterilized at 121°C for 15 min.

Preparation of the crude extract

Bacterial cells were harvested by centrifugation at 10,000 rpm for 20 min then washed by 150 mM potassium phosphate buffer (pH 8.0) after that the cells were resuspended in the same buffer. The resuspended cells were disrupted by ultra-sonication to give the crude enzyme extract.

Assay of D-hydantoinase

The enzyme assay was carried out according to Niu *et al.* (2007) with some modifications. D-hydantoinase activity was estimated by adding 2.0 ml of D-hydantoinase solution to 1.0 ml of D-hydantoin 2.0 g/l in 150 mM potassium phosphate buffer (pH 8.0). The mixture was incubated at 40°C with constant shaking for 10 min. The reaction was then terminated by adding 0.5 ml of 5% $ZnSO_4$ solution followed by centrifugation at 5,000 rpm and the supernatant was used for the *N*-carbamoyl-D-amino acid assay according to Morin *et al*. (1987).

The assay included 1 ml of the reaction mixture supernatant, 0.5 ml Ehrlich's reagent (dissolve 250 g *p*-dimethylaminobenzaldehyde in 250 ml 6 N HCl and 2.5 ml distilled water. The concentration of the *N*-carbamoyl-amino acid produced can be calculated using a standard curve of *N*-carbamoyl-amino acid subjected to the Ehrlich's assay.

Effect of surfactants

The effect of the Triton X-100 and Tween-20 as surfactants on D-hydantoinase was investigated. The crude enzyme preparation was treated with Triton X-100 or Tween-20 at various concentrations (1, 2, 3, 4 and 5% v/v) followed by the enzyme assay.

Determination of immobilization yield for D-hydantoinase

The immobilization yield of the enzyme was defined as the yield of the enzyme immobilized in alginate bead and expressed as:

Immobilization yield (%) = (Activity of immobilized enzyme/ Activity of the added enzyme) x 100

Influence of adenosine triphosphate (ATP) on D-hydantoinase activity

The enzyme activity of D-hydantoinase was assayed in presence of various concentrations (2, 4, 6, 8 and 10 mM) ATP in the reaction mixture.

Effect of chelating agent on D-hydantoinase activity

The chelating agents hydroxyquinoline, EDTA, α,α-dipyridyl and *o*-phenanthroline were tested regarding their effect on D-hydantoinase activity. They were tested at 2, 4, 6, 8 and 10 mM.

Influence of metal ions on D-hydantoinase activity

Eight metal ions were tested in the present investigation. They were Cu^{2+}, Mn^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}, Co^{2+}, Ca^{2+} and Ni^{2+}. These metal ions were tested in the reaction mixture as chloride salts at either 1 mM or 5 mM. The D-hydantoinase activity was measured as mentioned previously.

Enzyme immobilization on alginate bead

The method was initially adopted from that of Foster *et al*. (2003). The pure D-hydantoinase was added to 40 ml sodium alginate (2.5% w/v). The solution was placed in a separating funnel and suspended over a beaker containing 200 ml % (w/v) $CaCl_2$. The alginate beads were then prepared by dropping gently the alginate solution through a 200 µl Eppendorf tip into $CaCl_2$ solution at a rate of approximately 30 drops min^{-1}. After 3h stirring gently

to allow the bead to harden, the bead was filtered out of the $CaCl_2$ solution and thoroughly washed by the same buffer. The activity of the immobilized enzyme was assayed.

Statistical analysis

All the data in the present study are expressed as mean ± SE obtained from three measurements.

Result and Discussion

Integral membrane proteins can only be released from membranes by detergents or chaotropic agents, which disrupt the membrane structure. This is because they have hydrophobic peptide chains that partially or completely cross the lipid bilayer (De Lima Santos and Ciancaglini, 2000). Therefore, the crude enzyme extract was treated with non-ionic detergents in order to solubilize the cell membranes and to evaluate the effect of this on the solubility of the enzymes.

Detergents are able to displace the protein, which is tightly bound by hydrophobic forces within a membrane by dissolving the membrane and replacing the membrane by aliphatic or aromatic chains which form part of the detergent (Scopes, 1987).

Triton X-100, as a non-ionic detergent, is widely used for this purpose, because it is mild in its action and therefore, solubilizes proteins without denaturing them. Many anionic detergents are extremely denaturing therefore, they are not useful for isolation of enzymes (Neugebauer, 1988; Bollag and Edelstein, 1991).

The D-hydantoinase activity in the enzyme preparation was assayed in the presence of various concentrations (1, 2, 3, 4 and 5 v/v %) of Triton X-100. The level of D-hydantoinase activity was increased remarkably in presence of Triton X-100 (Fig. 1). The optimal level of Triton X-100 for the solubilization of the protein was observed at 3% for the hydantoinase where the activity was 43 units per mg protein.

Also, the solubilizing effect of Tween-20 at various concentrations (1, 2, 3, 4 and 5 v/v %) on D-hydantoinase activity was tested. The results in Fig. 1 show that there was continuous increase in the enzyme activity up to 4 v/v % after which the enzyme activity decreased.

Thus, both of Triton X-100 and Tween-20 at their lower concentrations increased D-hydantoinase activity from *B. theorgensis.* The solubilizing effect on D-hydantoinase in the present work was a significant result, indicating that the enzyme may be integral or anchored membrane protein. This property of the enzyme from *B. theorgensis* has been reported also for hydantoinase enzymes from *Agrobacterium* sp. and *Pseudomonas* sp. (Burton *et al*., 1998).

D-hydantoinase was purified from *B. theorgensis* using ammonium sulphate (55-75%), phenyl sepharose and sephacryl S-200 with specific activity of 201.7 units per mg protein and 16.5-fold (Data not shown). The pure enzyme preparation was used in the next experiments.

The effect of ATP on D-hydantoinase activity was investigated at various concentrations (2, 4, 6, 8 and 10 mM). The results in Fig. 2 indicate continuous increase of the activity in concentration-dependent manner up to 8 mM after which there was little increase.

These results reveal that the enzyme from *B. theorgensis* may be dependent on ATP. ATP-dependent D-hydantoinase has been reported by other investigators (Ishikawa *et al.*, 1994; Ogawa *et al*., 1995a). Thus, Mg^{2+} may be involved in the functioning of the enzyme. In support, the activities of several ATP-independent hydantoinases have been enhanced by the presence of Mg^{2+} ions (Buson *et al*., 1996; Sharma and Vohra, 1997; Soong *et al.*, 1999).

The metal dependence of D-hydantoinase from *B. theorgensis* was investigated by adding ethylenediaminetetraacetate (EDTA), *O*-phenanthroline, α,α-dipyridyl or hydroxyquinoline individually to the enzyme preparation at various concentrations (2-10 mM). These compounds were added in the reaction medium as metal ion chelators to remove metal ions bound within the enzyme followed by enzyme assay.

The results in Fig. 3 show that the four tested compounds inhibited the enzyme activity and the inhibition was dependent on both the concentration and the compound. EDTA was the weakest inhibitor; however hydroxyquinoline was the strongest one. C_{50} values were 6.5, 4, and 3.3 mM for *o*-phenanthroline, α,α-dipyridyl and hydroxyquinoline, respectively. The weakest chelating ability of EDTA compared with *o*-phenanthroline, α,α-dipyridyle and hydroxyquinoline was also reported by Huang *et al.* (1999).

The inactivation of D-hydantoinase in the present investigation by *o*-phenanthroline, α,α-dipyridyle and hydroxyquinoline is in harmony with the results of Zhang *et al.* (2010). This inactivation seems to be related to chelating of some metal ions.

The effect of metal ions on D-hydantoinase activity was investigated. The metals were used as chloride salts. These cations were Cu^{2+}, Mn^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}, Co^{2+}, Ca^{2+} and Ni^{2+}. They were tested at 1 mM and 5 mM and the

results are shown in Fig. 4. D-hydantoinase was activated by Ca^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+} particularly at 5mM. However, both Cu^{2+} and Fe^{2+} were inhibitors.

Ca^{2+} proved to be the best activator for D-hydantoinase from *B. theorgensis*. Ca^{2+} stimulated D-hydantoinase activity from *Bacillus* sp. AR9 (Kishan *et al.,* 2005). Also, D-hydantoinase was activated by divalent cations such as Co^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} (May *et al*., 1998a,b).

Previous reports have revealed that Zn^{2+} plays an important role in the catalytic process of D-hydantoinase, and in fact most D-hydantoinases are Zn^{2+}-enzymes or Zn^{2+}-dependent enzymes (Jahnke *et al.*, 1993; Abendroth *et al.,* 2002). Metal-dependent D-hydantoinases (Lee *et al.*, 1995; Park *et al.,* 1998) and L-hydantoinases (Ogawa *et al.*, 1995b) requiring Mn^{2+}, Mg^{2+} and K^{+} have been reported. The activity of hydantoinase from *Agrobacterium* sp. has been enhanced by Ni^{2+} or Mg^{2+} (Runser and Ohleyer, 1990).

The inhibition of D-hydantoinase from *B. theorgensis* by Cu^{2+} and Fe^{2+} is in harmony with those reported by Shi *et al*. (2006) for the enzyme from a recombinant *E. coli.*

The effect of various concentrations of sodium alginate on immobilization yield of D-hydantoinase from *B. theorgensis* was investigated. Sodium alginate was tested at 1, 2, 3, 4 and 5 % (w/v). The results in Fig. 5 reveal that increasing sodium alginate up to 3% (w/v) resulted in continuous increase of immobilization yield. However, any further increase in sodium alginate concentration caused reduction in the yield.

The effect of immobilization time on immobilization yield of D-hydantoinase was studied. The results in Fig. 6 show that the immobilization yield increased with the time up to 5h after which the yield decreased. In solution, intramolecular bonds between protein molecules could result in inactive aggregates.

The effect of various concentrations of $CaCl_2$ in the alginate bead on the immobilization yield of D-hydantoinase was tested. $CaCl_2$ was tested at 1, 2, 3, 4 and 5 % (w/v). The results in Fig. 7 indicate that increasing of $CaCl_2$ concentration up to 3% (w/v) resulted in the increase of immobilization yield after which the yield was declined.

The influence of potassium phosphate buffer (pH 8.0) and Tris-HCl buffer (pH 8.0) on immobilization yield of D-hydantoinse was studied. Each buffer was tested at various concentrations (50, 100, 150, and 200 mM). The result in Fig. 8 show that 150 mM potassium phosphate buffer was better than Tris-HCl buffer for enzyme activity.

It seems likely that immobilization yield of D-hydantoinase was affected by the type of buffer used. Similar variations in the hydantoinase activities have been observed with the use of these buffers (Möller *et al.,* 1988; Sharma and Vohra, 1997; Sudge *et al.,* 1998).

In conclusion, D-hydantoinase activity was enhanced by Triton X-100 and Tween-20 revealing that the enzyme is particulate. Also, the enzyme was successfully immobilized on alginate bead with appreciable yield and this of significant importance since this enzyme is involved in many industrial applications.

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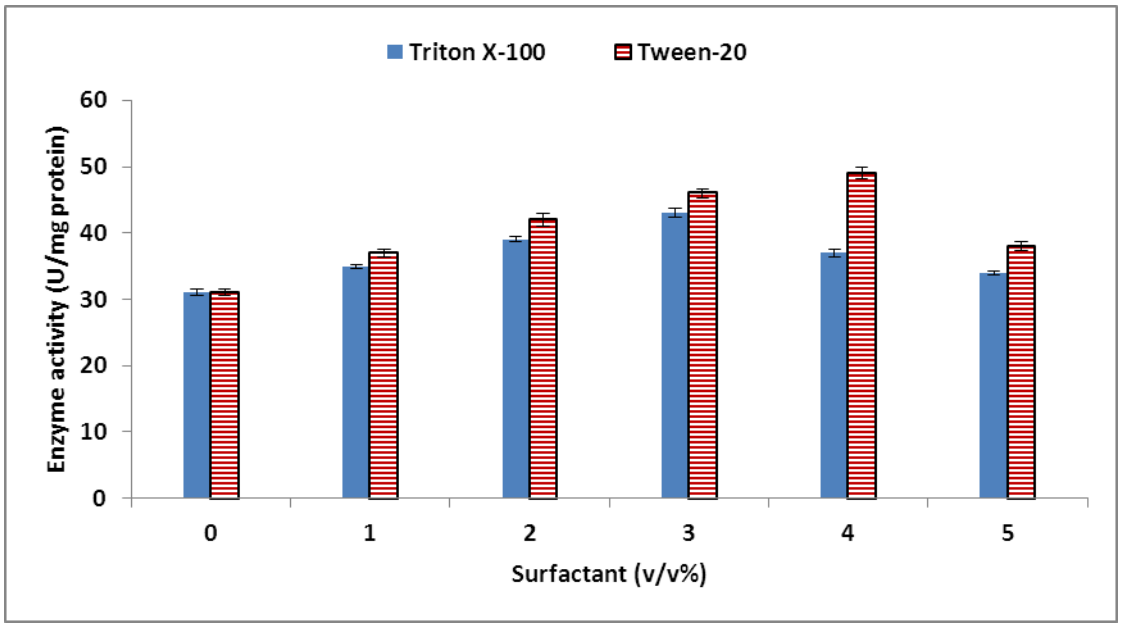

Fig. 1: Effect of Triton X-100 and Tween-20 on enzyme activity from *B. theorgensis.*

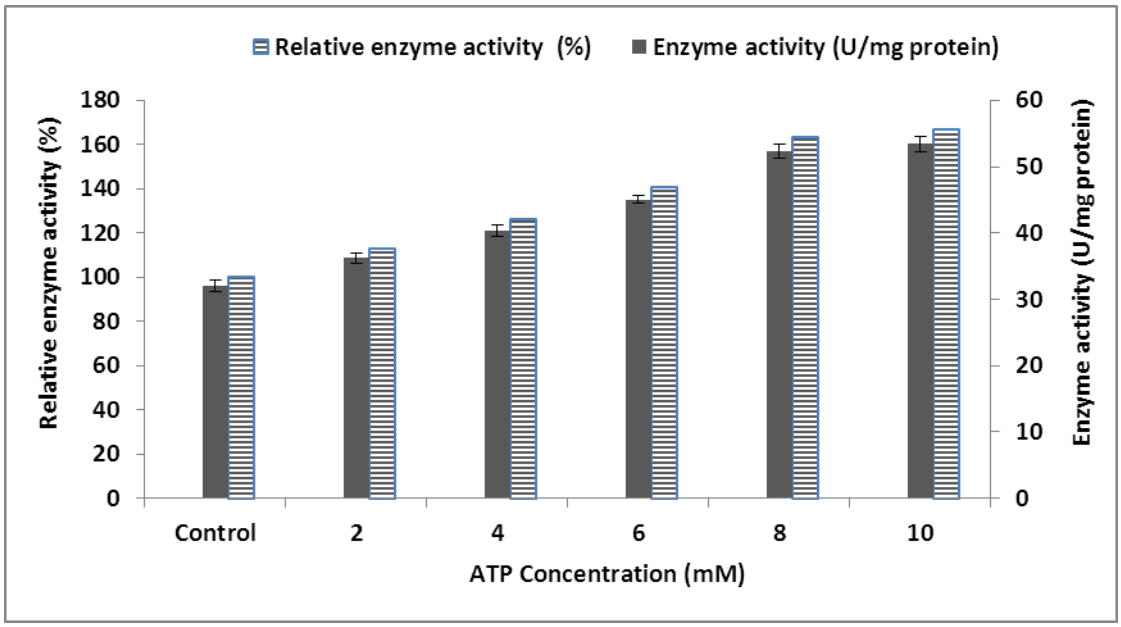

Fig. 2: Influence of ATP on D-hydantoinase activity from *B. theorgensis*.

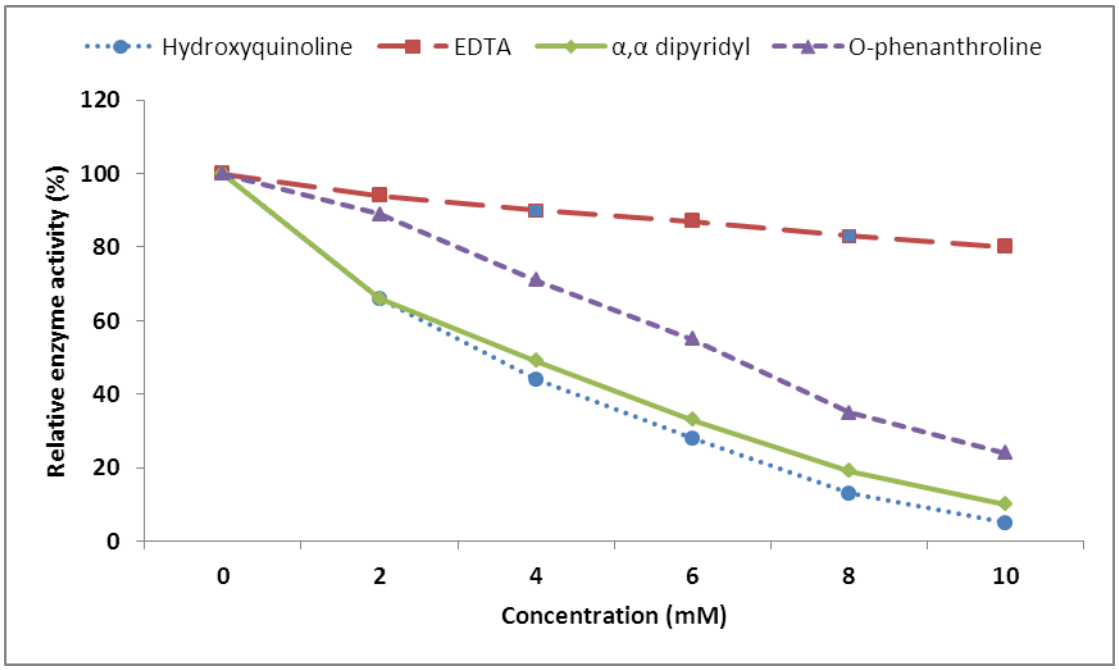

Fig. 3: Effect of chelating agents on D-hydantoinase activity from *B. theorgensis.*

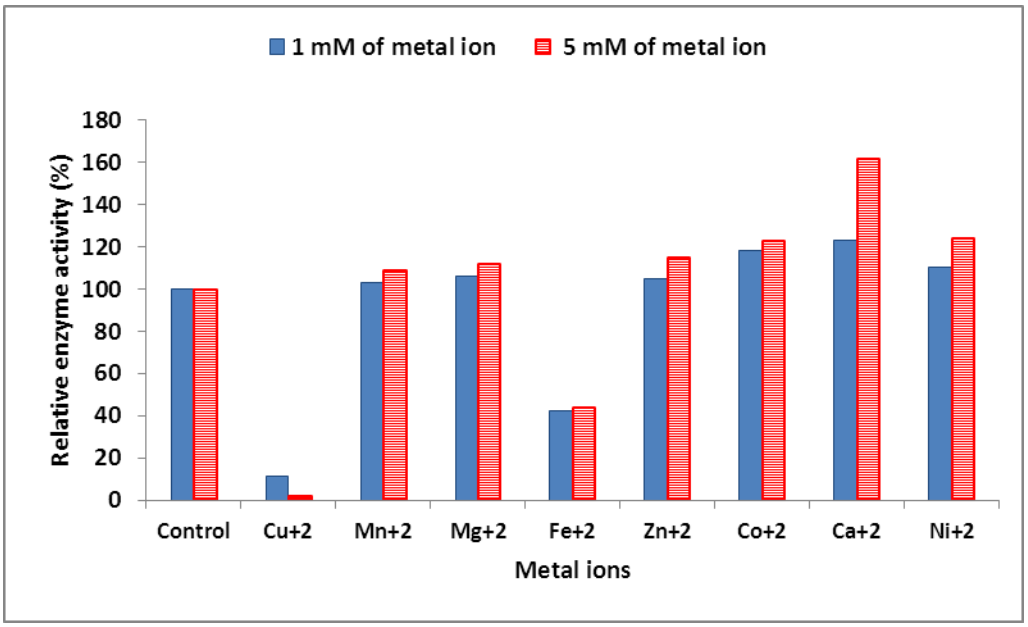

Fig. 4: Influence of metal ions on D-hydantoinase activity from *B. theorgensis.*

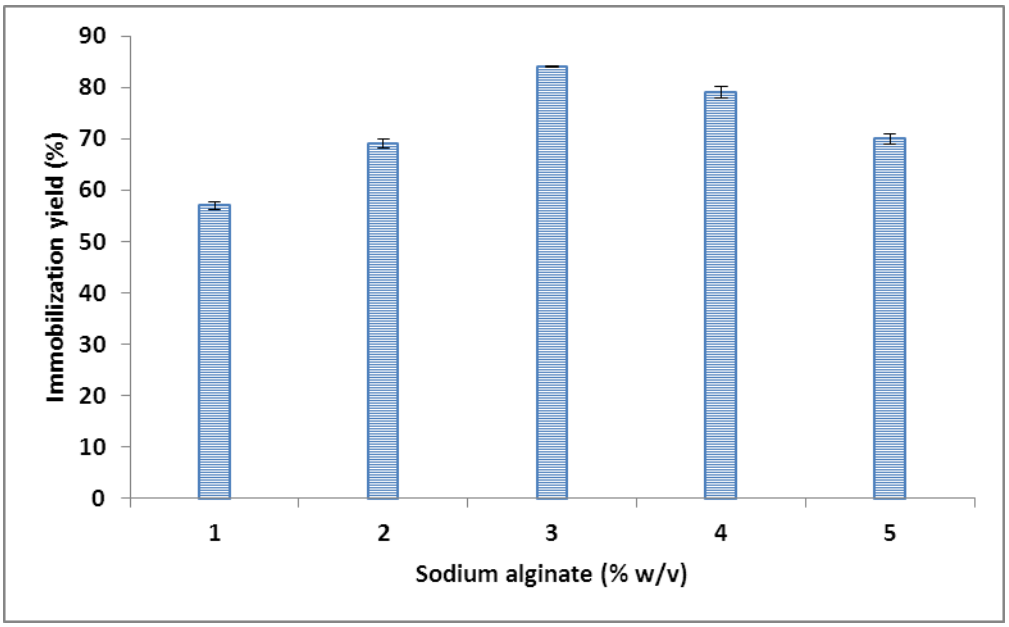

Fig. 5: Effect of sodium alginate concentration on immobilization yield of D-hydantoinase from *B. theorgensis.*

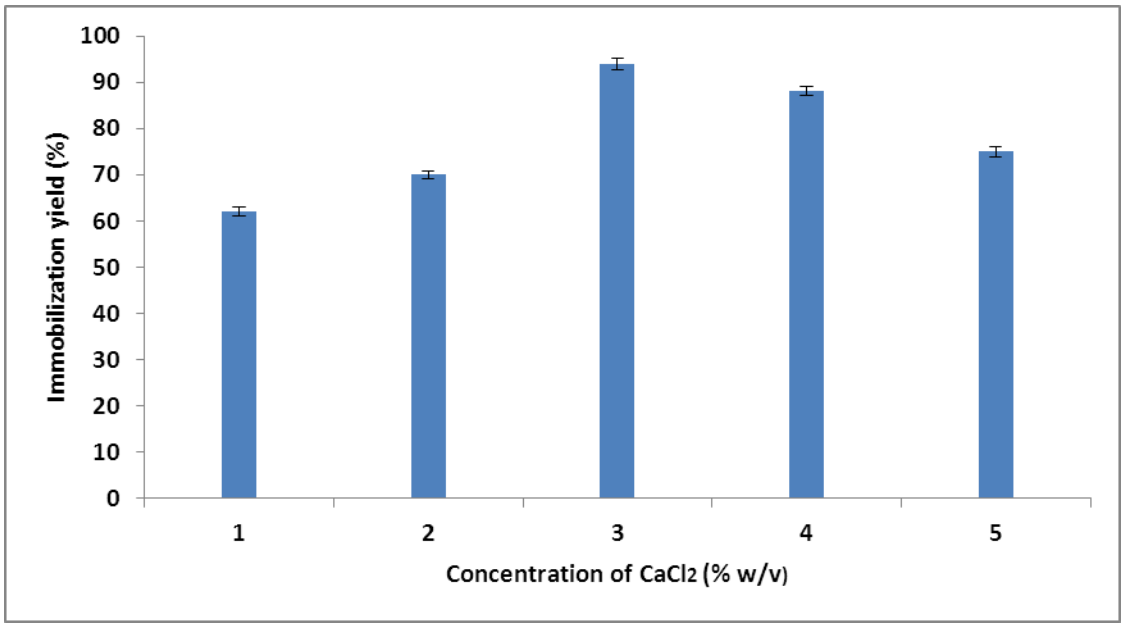

Fig. 6: Effect of $CaCl_2$ on immobilization yield of D-hydantoinase from *B. theorgensis.*

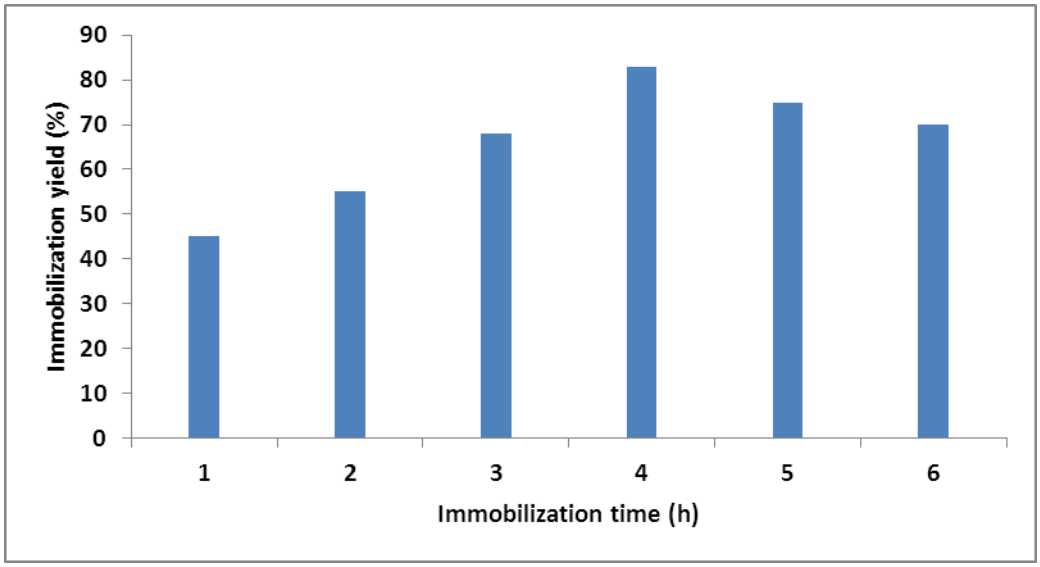

Fig. 7: Effect of immobilization time on immobilization yield of D-hydantoinase activity from *B. theorgensis.*

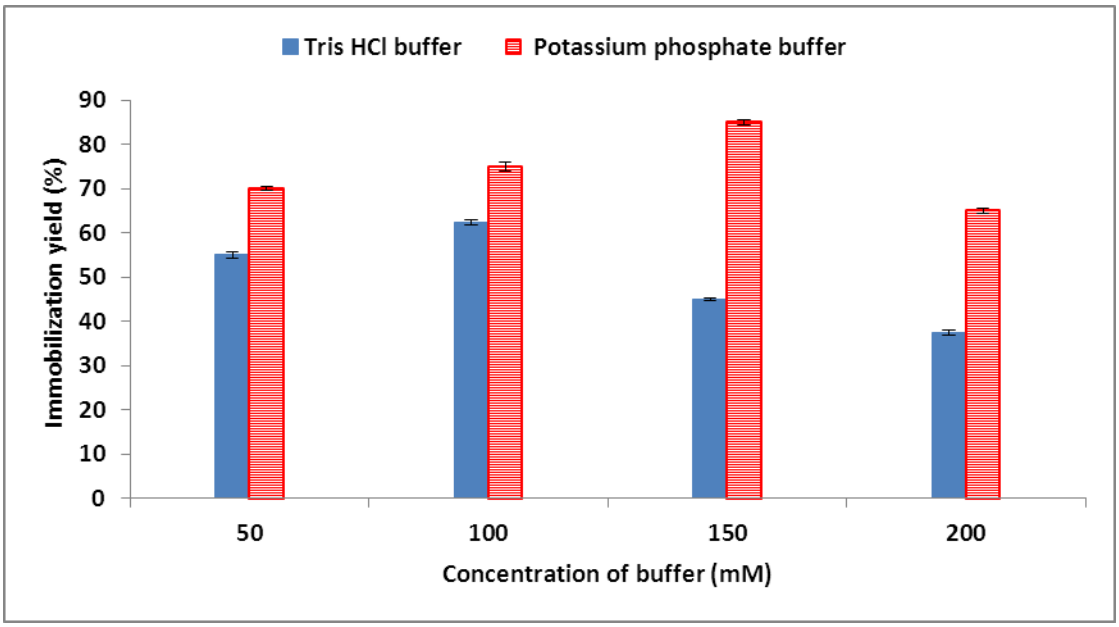

Fig. 8: Effect of buffer concentration on immobilization yield of D-hydantoinase from *B. theorgensis.*