# Efficient immobilization of Diamine Oxidase by entrapment and study of optimum parameters

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The Presence of Diamine oxidase (DAO) has gained tremendous attention in food and allied industries for guarding food freshness and safety.All the commercial application of enzymes as biocatalysts requires simple but efficient methods to immobilize the enzyme to yield highly stable and active biocatalysts. In this study, DAO was extracted, purified and immobilized by entrapment in matrix sodium alginate in presence of the enzyme. The optimal parameters of free and immobilized DAO activity were evaluated against the substrate, Putrescinedihydrochloride and the results showed that the stabilities were significantly enhanced compared with free counterpart. The optimum working pH was 7.5 where both free and immobilized enzyme showed their maximum activity.. Free enzyme showed maximum activity at 37°C while immobilized enzyme showed stable activity at a wide range of temperature. Residual activity of the immobilized enzyme was 59% of the initial activity after being recycled 10 times. Therefore, this method may provide an excellent support for enzyme immobilization having better catalytic ability in large scale applications in biotechnological industries.

**Keywords: Diamine oxidase enzyme; entrapment immobilization, Comparative Study of Enzyme properties**

**Diamine oxidase** is an intracellular enzyme found in submucosal villi of human and mammalian small intestine, it play important role in histamine and polyamine metabolism and also catalyze oxidation of putrescine, & diamines to aldehyde. Diamine oxidase is found widely in plant tissues, animal tissues and microorganisms. Diamine oxidase is water soluble and it has been crystallized in ruminant plasma. Diamine oxidase which is found in human are heterodimer containing two subunits.

Highest level of expression of diamine oxidase is found in digestive tract and placenta. In pregnant women trophoblastic cells of placenta release diamine oxidase and release it into blood. Although diamine oxidase is found rarely in blood circulation, but during pregnancy release in diamine oxidase concentration in blood indicates it protect against bad histamine. Diamine oxidase is also released by eosinophils. Excretion of diamine oxidase occurs through bile and urine. Uptake and excretion of diamine oxidase is suppressed when liver function is damaged which leads to increased concentration of serum diamine oxidase. In case of

intenstional mucosal disease concentration of diamine oxidase decrease. Therefore concentration of diamine oxidase in serum and tissues can be used to determine disease in small intestine (Mcgrath *et al.,* 2009). Diamine oxidase can be immobilized by different methods.

Diamine oxidase has been used widely for diagnostics purpose. Activity of diamine oxidase increases during asthmatic attack. Histamine intolerance in patients can be diagnosed by measuring the diamine oxidase activity and migraine patients are also reported to have low level of serum diamine oxidase. For diagnosis purpose diamine oxidase can be immobilized by different methods. Immobilization of diamine oxidase for diagnosis is very useful because enzyme can be reused for diagnosis and it will be cheap method if enzyme can be reused.

Immobilized enzymes are used for diagnosis, disease treatment (to treat inborn metabolic disorders) and also used as medicines (Tischer & Wedekind 1992). Immobilized enzymes have several benefits over free enzymes because free enzymes are used by cells and can't be used for long time but immobilized enzymes are not consumed by cells, remains stable for long time and have prolonged use.

To treat cancer with immobilized enzymes, enzymes are delivered to oncogenic sites. To deliver enzymes as therapeutic agents at their target site, usually nanoparticles and nanospheres are used as carrier. Immobilized enzymes can also be used for syrup production, immobilized beta- galactosidase is used for lactose hydrolysis for baker's yeast production. Production of liquid fuels with immobilized lipases is used nowadays (Fjerbaek et al., 2009), lipases production is very costly and hence immobilization of lipases results in repeated use and stability.

We know production of enzymes by biotechnological production is expensive, hence new methods are developing to reduce cost of their production. Other problems associated with enzyme purification are low stability and high sensitivity to process conditions, and all these problems can be overcome by using immobilization technique (Cao et al., 2005; Hernandez & Fernandez-Lafuente et al., 2011; Krajewska et al., 2004). Enzyme immobilization fixes the enzymes and thus restricts the movement of an enzyme. Immobilization reduce the cost of processing, enzyme can be reused, cost of enzyme and resulting product also decreases and immobilized enzymes are more stable than dissolved enzymes (Krajewska *et al.,* 2014). Enzyme immobilization can be done by different methods.

## Principal methods for immobilization of enzyme

- ***Adsorbtion***- Adsorbtion involves reversible surface interactions between carriers on which enzyme which will be adsorbed and enzymes (Wahba & Hassan 2015). Surface interaction occurs by mostly weak forces like electrostatic , Vander waals forces, hydrogen bonds and ionic bond interactions.
- ***Covalent Bonding***- This method involves covalent bond formation between enzyme an carrier (Toher et al., 1990; Porath & Axen 1976) and bonding occur between functional groups of both enzymes and carrier.
- ***Entrapment***- Entrapment involves cross-linking of polymers (e.g. Polyacrylamides, silica gel and silica gel) in presence of enzyme and enzymes will get trapped in polymer.
- ***Encapsulation***- In this method support material containing affinity ligand is activated and then enzyme is added. In this method enzyme is not exposed to harsh chemical conditions.
- ***Cross Linking***- This method is matrix free, support is provided by cross linking of enzymes by physical or chemical methods which provide three dimensional complex structures (Ahmed et al., 2016).

## Diamine oxidase (DAO)

Diamine oxidase (DAO) is an enzyme also called histaminases which is involved in metabolism, oxidation and inactivate histamine and other polyamines such as putrescine, spermidine, ethylene diamine and other diamines of general formula $NH_2(CH_2)_nNH_2$ where n= 2 to 8 to aldehyde. DAO catalyze oxidative deamination of amino acids according to following general equation:-

$$R\text{-}CH_2NH_2 + O_2 + H_2O \xrightarrow{DAO} R\text{-}CHO + H_2O_2 + NH_3$$

Some properties of DAO are:-

- **Structure**

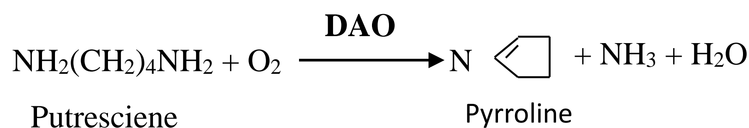
The molecular weight of DAO from clover is 144 KD (Delhaize and Webb, 1987) while that from soybean is 225 KD, 184 KD for the pea enzyme and 150 KD for lentil enzyme. DAO is a homodimer consist of two identical subunits with each subunit has molecular mass 87 kDa and are linked by disulfide bond (Yanagisawa *et al.*, 1981). Each subunit of DAO contains one molecule of pyridoxal phosphate and one atom of copper. DAO contain $Cu^{2+}$ as prosthetic group which can be easily removed from enzyme by dialysis against chelating agents which leads to enzyme inactivation, but addition of small amount of $Cu^{2+}$ can restore the activity of enzyme. This property of enzyme can be used as highly sensitive method for detection of $Cu^{2+}$ even in small quantities. DAO catalyze oxidative deamination of primary amino group into aldehyde.
DAO is pink colored, even in the absence of copper, but it becomes yellow on adding substrates in the absence of oxygen . Cofactor of DAO was thought to be pyridoxal phosphate on the basis of phenylhydrazine-enzyme adduct. However later it was found active cofactor in enzyme is pink color pyrroloquinoline quinine (PQQ) (). In mammalian DAO redox cofactor was found to be 6-hydroxydopa (Janes et al., 1990) which is similar to pyrroloquinolone. Later 6-hydroxydopa was also found in pea enzyme.

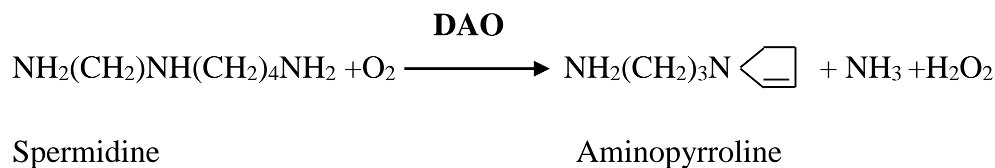
- **Distribution**

DAO is found in many plants (Smith 1980) but mostly found in leguminosae like from pea seedlings (Hill, 1971), soyabean radicles, pea cotyledons (Tajima *et al.*, 1985), in cereal embryos and in tubers of *Helianthus tuberosus* (Torrigiani *et al.*, 1989).
Pea seedlings are a rich source of DAO and remains stable during long term storage and assays due to which pea seedlings can be used for estimating amines like lysine and arginine by using membrane bound DAO with an oxygen electrode. DAO in pea seedlings is found mostly in cotyledons and after 8-16 days of germination in dark concentration of DAO reaches to peak (Kenten & Mann, 1952; Hill & Mann, 1968). Pretreatment of pea seedlings with putrescine, spermidine, or ornithine causes DAO level to increase in seedlings. (Srivastava *et* al., 1977). Phytic acid also stimulates activity**.** In case of animals highest level of expression of DAO is found in placenta and digestive tract. In case of pregnant women extravillous trophoblasts cells of placenta express DAO and release DAO into blood stream. Low level of DAO in blood during pregnancy indicates trophoblast related pregnancy disorder. Level of DAO increase during pregnancy protect against adverse effect of histamine. In case of low level of serum DAO concentration disorders like allergies and histamine intolerance may occur. Intravenous injection of heparin increases the serum DAO concentration.

## ➢ Mechanism of Action

DAO oxidizes spermidine to pyrroline and spermidine to aminopropylpyrroline with production of diaminopropane and hydrogen peroxide:




Presumed aminopropylpyrroline synthesized from spermidine by the oxidative method of with metallic copper in alkali as a catalyst, was subsequently shown to have a bicyclic structure.

## ➢ Purification

Crystallization and electrophoresis are two main methods for enzyme purification. But these methods are not used for large scale purification, used only at laboratory scale. At laboratory scale chromatography is of fundamental importance. Enzymes are separated on the basis of their physical property (shape, size, hydrophobic interactions, charge), biological properties (biospecific affinity) or chemical properties (covalent binding). Now a day's industry uses ultra filtration to produce enzymes.

**DAO Purification**:-

DAO purification is done by following methods

- Centrifugation
- Precipitation

Extraction

- Electrophoresis
- Chromatography
- Ultrafiltration

## ENTRAPMENT IMMOBLIZATION

## Material method

## Chemical and reagent:

Lab purified Diamine oxidase enzyme, glutaraldehyde (2.5%), 10% TCA [illegible] thanol, acetic acid from Merck life sciences Pvt. Ltd. Mumbai, 0.1M Potassium phosphate

buffer (pH7.2), potassium dihydrogen phosphate ($KH_2PO_4$), potassium phosphate dibasic ($K_2HPO_4$), Putrescine as substrate from HIMEDIA Lab. Pvt. Ltd., Ninhydrin reagent, Folin's reagent, sodium alginate, calcium chloride, SODIUM ALGINATE, Double distilled water (DW) was used in all experiments.

## 3.2 Instrumentation:

Centrifuge, digital pH meter (EUTECH), weighing machine (METTLER TOLEDO), water bath (SUNRON), incubator, UV/Vis spectrophotometer (SYSTRONICS), cold centrifuge superspin, pestle and mortar ,sieve, syringe needle, muslin cloth, quartz cuvette, vortex, shaker, magnetic stirrer (HICON), glasswares (all from Borosil glass works ltd. Mumbai).

## Collection of plant material and growth conditions:

Certified *Pisum sativum* seeds were purchased from a local dealer. The seeds were washed thoroughly with tap water and then soaked in sufficient water and incubated in a dark place overnight at room temperature. Next day, seeds were transferred to the mud trays for germination and sprouting. Literature confirmed that the highest efficiency of DAO in pea seedlings is seen on days 7-8 of growth in the dark. Therefore, the tray was kept in complete dark place for a week. On day 8, germinated seedlings were used for enzyme extraction procedure.

## Enzyme extraction from Pea seedlings:-

The pea seedlings were washed by distilled water to remove soil and any other debris. Weighed seedlings were homogenized with 0.02 M phosphate buffer solution (pH 7.2) and cold water in a homogenizer and squeezed through muslin cloth to obtain a crude homogenate . The obtained extract was collected and quantified for its total protein concentration and DAO activity.

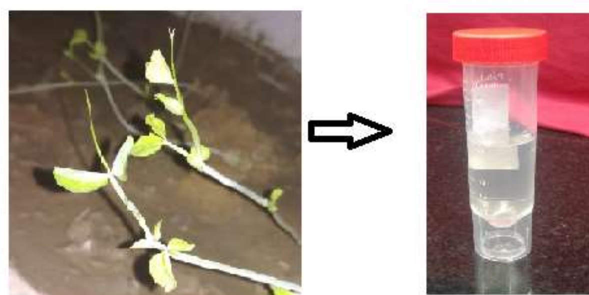


Fig.Crude extraction of DAO from pea seedlings

## Total Protein estimation:-

Total protein concentration in the crude extract was estimated by Lowry's Method (Lowry *et al*, 1951) using BSA as a standard protein.

**Principle:-**

The principle lies in the reactivity of the peptide nitrogen[s] with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin–Ciocalteu reagent (FCR) (or Folin's phenol reagent or Folin–Denis reagent) phosphomolybdic phosphotungstic acid to heteropolymolybdenum blue coloured complex by the copper-catalyzed oxidation of aromatic acids (by tyrosine and tryptophan residues present in protein) which absorb maximum at wavelength 660-750 nm.

**Preparation of reagents:-**

The following reagents were used in the method:

**Reagent A :** 2% $Na_2CO_3$ in 0.1 N NaOH

**Reagent B1:** 1% Copper sulphate in distilled water, stored at 4ºC

**Reagent B2:** 2% NaK Tartrate in DW, stored at 4ºC

**Reagent B :** B1+ B2 in 1:1 ratio, prepared freshly

**Reagent C :** Reagent A + Reagent B in 50:1 ratio, freshly prepared at the time of use.

**Reagent D :** Folin–Ciocalteu reagent(2N) and distilled water in 1:1 raio. It was also prepared freshly.

**BSA Standard** - 1 mg/ ml

**Procedure:-**

Took 0.1 ml of protein solution and added 0.4 ml of DW. Then 5 ml of reagent C was added, mixed well and allowed to stand at room temperature for 10 minutes.

Then 0.5 ml of reagent D was added and mixed thoroughly with vortex. This solution was allowed to stand at room temperature for 30 minutes. The intensity of the colour developed was measured at 750 nm in UV/Vis spectrophotometer (SYSTRONICS). The amount of protein was calculated from standard curve of Bovin Serum Albumin (BSA). The blank was prepared by mixing 0.5 ml DW, 5 ml of reagent C and 0.5 ml of reagent D.

**Preparation of standard curve of BSA:-**

To prepare std. curve of BSA, different conc. of BSA solutions such as 1mg/ml, 2mg/ml, 3mg/ml, 4mg/ml, 5mg/ml, 6mg/ml, 7mg/ml, 8mg/ml, 9mg/ml, 10mg/ml were prepared from the stock solution of BSA (1 mg/ml). Took 0.1ml of the following BSA dilutions and the above procedure was followed for protein determination. A curve was plotted between absorbance at 750nm and different BSA concentrations the standard curve shown in fig.9 .

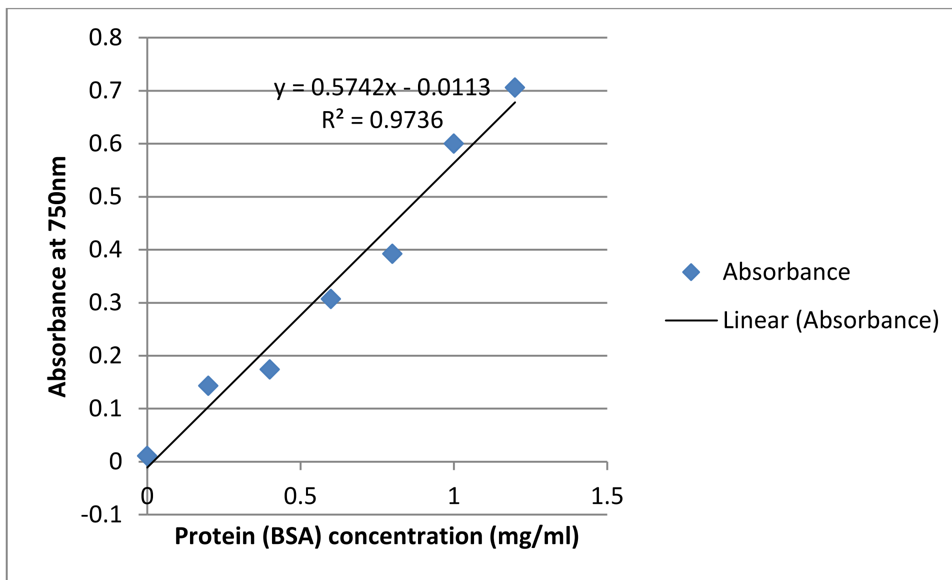



Fig. Standard curve of BSA

## Spectrophotometric analysis of DAO activity (Enzyme assay):-

Diamine Oxidase was assayed by the method of Naik *et al.,* (1981).

The assay system for DAO contains putrescine, 5 µmol; Tris-HCl buffer pH 7.0, 50 µmol; and enzyme 0.2 ml. After incubation for 30 min at 37˚C, the reaction was stopped by adding 0.5 ml of TCA(10%). After termination of the reaction substrate was added in the control tubes. The ninhydrin method of Naik *et al.,* (1981) was preferred to estimate the pyrroline formed.

**Estimation of A-pyrroline**:

From the assay tubes a small portion of the centrifuged supernatant was taken and its volume made up to 1 ml. Then warm ninhydrin reagent 1.0 ml (made by mixing 250 mg of ninhydrin and 37 mg of hydrindantin in 4.ml of 6.0 M o-phosphoric acid and 6.0 ml of acetic acid) was added to it followed by 1.5 ml acetic acid. The tubes were placed in a boiling water bath for 30 min to get the colour. Then the tubes were cooled and added 2.5 ml acetic acid, made up the volume to 6.0 ml. The intensity of the colour was measured at 510 nm spectrophotometrically.

**Preparation of hydrindantin**: Took 4 g of ninhydrin and dissolved in 100 ml of DW and heated to 90 °C. Then added ascorbic acid solution (4g/ml) to it with stirring. The crystalswere formed, cooled them, filtered and washed with distilled water (DW), and dried and kept in a brown bottle.

**Enzyme unit**: Enzyme unit is is defined as the concentration of enzyme required for the converting 1 mol of substrate per second.

## Purification of pea seedlings:-

Purification focused on to isolate the desired enzyme i.e, DAO with maximum possible yield. Following steps were followed to purify DAO as shown in fig.10 .

**Centrifugation:**

The crude homogenate was centrifuged at 12,000 rpm for 30 minutes at 4ºC to remove the heavy suspended insoluble particles or debris in the suspension, Then the supernatant (230 ml) was collected for the next step.

**Ammonium sulphate precipitation:**

The ammonium sulphate was added slowly to the collected supernatant at a fixed rate with constant stirring on a magnetic stirrer. It was added to 60% saturation. This made proteins insoluble and precipitates were formed due to high salt concentration (salting out). This high salt concentration changed the ionic stability of the proteins and decreased available water for proteins to solubilize.

**Extraction:**

After extraction, DAO enzyme solution was centrifugrd again at 10,000 rpm for 20 min. at 4ºC. The pellet was collected and resuspended in 0.02 M PB and left overnight at 4ºC.

Next day, the pellet suspension was centrifuged and supernatant cantaining DAO enzyme was taken.

**Dialysis:-**

After precipitation and extraction, the high amount of $(NH_4)_2SO_4$ was reduced before proceeding to the next step because it might denature the enzyme. In this the partially purified extract was fiiled in a bag of NC membrane. Then, this bag was immersed in a large volume of buffer. This is placed on a stirrer for 5-6 at about 4ºC. The buffer was changed after a certain period of time intervals to dilute the enzyme solution.

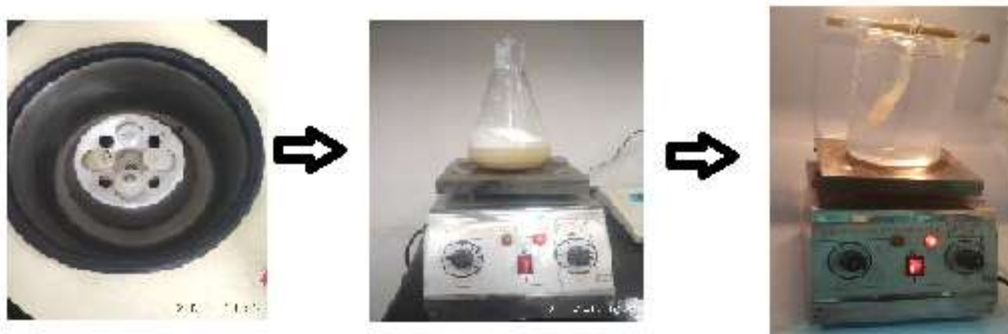


Fig. Purification of DAO

**Electrophoresis:-**

Then SDS PAGE was performed. Solutions and buffers for this were prepared as follow:

**Preparation of reagents for SDS-PAGE:**

(a) **30% acrylamide/ bis-acrylamide gel**
(b) **1.5 M Tris-HCl buffer, pH 8.8**
(c) **1.0 M Tris-HCl buffer, pH 6.8**
(d) **10 % SDS –**
(e) **10% Ammonium Persulfate –**
(f) **Running buffer ( 1000 ml) –**

Dissolved 3g Tris base and 14.4 g glycine in 800ml distilled water. pH was adjusted to 8.5-8.7 and then add 0.1 g SDS. Raised the final volume upto 1000ml with DW.

**(g) Tracking dye–**

0.05% bromophenol blue dye solution was prepared in 1.5M Tris HCl buffer of pH8.8.

**(h) Separating gel preparation (7%) –**

It was prepared by mixing 3.45 ml dw, 2.5ml solution (a), 2.5 ml solution (b),50 µl of solution (d) and 5 µl of TEMED to make final volume 10 ml.

**(i) Stacking gel preparation (4%) –**

It was prepared by mixing 6.5 ml of dw, 2.5 ml of solution (c), 1.3 ml of solution (a), 10 µl of TEMED, 50µl of solution (d).

**(j) Staining solution-**

It was prepared by adding 0.5g Coomassie brilliant blue, 112ml Methanol, 25 ml Glacialacteic acid.

**(k) Destaining solution-**

It was prepared by adding 50 ml Methanol and 70ml Acetic acid and raised the volume upto 1000 ml with dw.

**(l) Sample preparation-**

0.02ml of enzyme was mixed with 0.02 ml of 20% glycerol containing 0.05% bromophenol blue with SDS.

**Procedure:**

1. The electrophoretic unit was set up and glass plates were assembled and spacers in between them. At first, separating gel solution was poured upto 70% in the space between the plates without formation of air bubbles. It was allowed to polymerize and solidify at room temperature.
2. Then the stacking gel solution was poured above the separating gel in the remaining space without formation of air bubbles.
3. Immediately, comb was fixed to form wells and allowed to polymerize.
4. It was assembled into the running chamber and poured enough running buffer so that it completely immersed in it. After solidification of gel, comb was removed.
5. Enzyme sample of about 50µl was loaded in wells with the pipette.
6. The apparatus was connected to electrophoretic power supply unit and run at 1.5mA/40V until the blue colour migrated just before to the lower end of separating gel.
7. The stopped the voltage supply and gel was removed and kept in staining solution for 1 hr.
8. Then gel was destained by keeping it in destaining solution for overnight. The gel is shown in Fig.11.

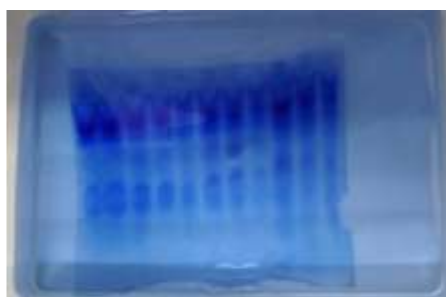


Fig. SDS PAGE gel

## Immobilization of the Enzyme:-

Enzyme immobilization by entrapment is a simple method. Sodium alginate beads are used for enzyme entrapment and also used in industries from a long time as a gelling, film forming, thickening and emulsifying agent. Entrapment by alginate beads is a fast, cheap and nontoxic method of enzyme immobilization.

## Materials

- Sterile sodium alginate for enzyme immobilization, sterilized by autoclaving.
- Solution of alginate 2-4% (w/v) prepared freshly each time when use in distilled water. Dissolution requires stirring.
- 3% calcium chloride solution is prepared.
- Phosphate buffer is prepared.
- Enzyme solution is prepare in phosphate buffer.

## Method

- Sodium alginate mixture containing the enzyme solution was transferred to a syringe that has mean of flow control.
- Place a beaker containing 1 L calcium chloride solution is put under the syringe and magnetically stir the solution to produce light vortex.
- Sodium alginate containing the enzyme mixture is added drop wise to calcium chloride solution from a height of about 10 cm.
- Sodium alginate mixture is added continuously until desired numbers of beads are formed.
- Before collecting the beads stirring is done for 20-30 minutes.
- Beads are washed with an appropriate buffer or distilled water to remove any residues of unbound enzyme.
- Beads are stored in minimal amount of calcium chloride solution.
- Dissolution of beads is done by incubating them at room temperature with phosphate buffer.( as shown in Fig. 12).

Fig. Entrapment in sodium alginate

## Comparative Studies of free and immobilized enzyme

### Effect of pH on DAO activity

The enzyme activity was determined at different pH (pH 5.5,6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0) at 37°C to observe the optimum pH.This response was measured by using different buffers each at 0.1M final of 0.1M PB.

**Effect of temperature on DAO activity**

The optimum temperature was measured by incubating the reaction mixture at $20^0$ C to $60^0$C at an interval of $5^0$C.

**Effect of incubation time on DAO activity**

The activity of the immobilized DAO was determined by assaying the enzyme after different time interval of incubation (10 to 45 minutes at an interval of 5 minutes) to study the effects of time period of incubation on enzyme activity.

In the present work, DAO enzyme from *P.sativum* seedlings was extracted, purified and immobilized onto sodium alginate as support. Enzyme was characterized with respect to its optimum pH, temperature, incubation time and substrate concentration.

## DAO extraction from pea seedlings (*P. sativum*)

In order to find out the best growth stage to obtain maximum DAO activity, specific activity of DAO in pea seedlings was measured as a function of sprouting seeds. Maximum activity was observed in 7-8 days germinating seedlings when grown in dark place.

## Purification of DAO enzyme:

Experimental results of the partial purification procedure of DAO are given in table 1. After the enzyme extraction from pea seedlings, the extracted enzyme solution was centrifuged and supernatant was treated with 60% $(NH_4)_2SO_4$ to purify DAO by altering its solubility. Total activity of the precipitate was 0.36 U/mg with 6 purification fold and 54 % yield. After this the enzyme was dialysed and final specific activity was calculated as 0.8 U/mg with 13-fold purification and 35% yield.

Table 1: Steps of purification of DAO

| **Step** | **Total protein(mg)** | **Volume (ml)** | **Specific activity (U/mg protein)** | **Total Unit (U)** | **Yield (%)** | **Purification, degree** |
|---|---|---|---|---|---|---|
| Tissue disruption and Filtration | 2150 | 340 | 0.06 | 115 | 100 | 1 |
| Centrifugation | 550 | 270 | 0.14 | 80 | 69 | 2 |
| Treatment with 60% $(NH_4)_2SO_4$ | 170 | 140 | 0.36 | 62 | 54 | 6 |
| Treatment at high pH, 24-h dialysis | 50 | 70 | 0.8 | 40 | 35 | 13 |

## Comparative Study of Enzyme properties :

Different parameters of DAO affixed on NC membrane and its comparison with those of free enzyme were observed. The following properties of the immobilized enzyme were studied-

### Effect of pH on DAO activity at 37°C:

This activity of free and immobilized DAO was determined between pH and  at an interval of 0.5 using glass electrode pH meter. The optimum working pH was 7.5 where both free and immobilized enzyme showed their maximum activity as shown in fig.13.

### Effect of incubation temperature:

The activity of the enzyme is also influenced with the fluctuations the temperature.The effects of temperature between 20ºC to 60°C. Initially the rate of reaction increases with increase in the incubation temperature but after a certain point, it decreases due to denaturation of the enzyme. Free enzyme showed maximum activity at 37°C while immobilized enzyme showed maximum activity between 35-40°C as shown in Fig.14.

### Effect of incubation time:

The activity of the immobilized DAO was determined by assaying the enzyme after different time interval of incubation to study the effects of time period of incubation on enzyme activity. The free enzyme showed maximum activity after 30 minutes of incubation while immobilized enzyme showed maximum activity after a wide range of incubation time as shown in fig.15.

### Storage and stability:

The immobilized DAO was stored at 4ºC in potassium phosphate buffer and it was found to be stable in activity for many times used but gradually lost activity after few days

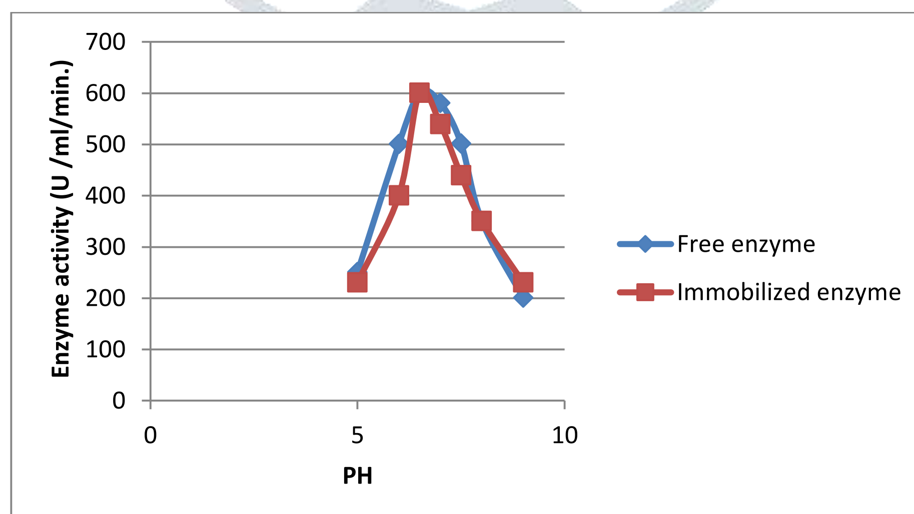



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Fig. Enzyme activity with varying pH

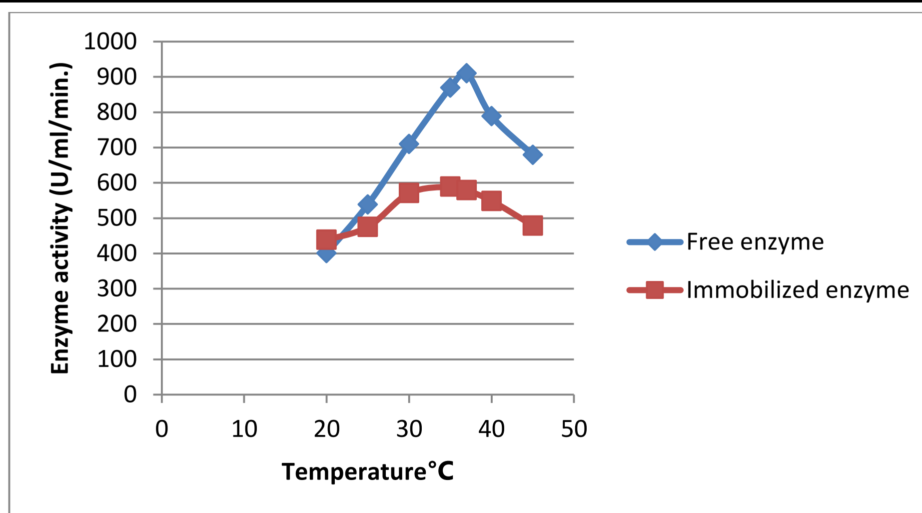


Fig. Enzyme activity with varying Temperature

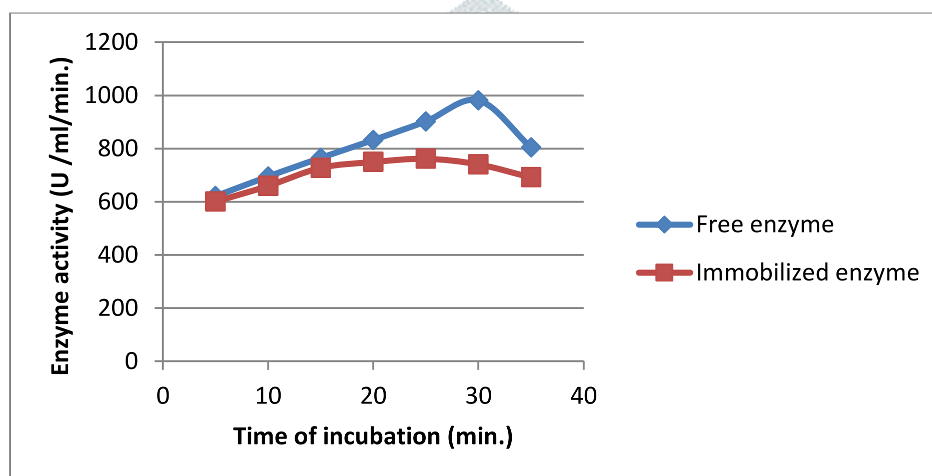


Fig. Enzyme activity with varying incubation time

Our study was to isolate and purify DAO from pea seedlings. Our aim was to obtain maximum possible yield and to immobilize it by entrapment method. DAO plays important role in DAO metabolism in plant, animals and microbes. DAO was successfully isolated and purified from 10 days old pea seedlings which were germinated in dark. . The final specific activity was calculated as 0.8 U/mg with 13-fold purification and 35% yield.

Purified DAO was then immobilized by entrapment method. Enzyme was entrapped in matrix sodium alginate. After that enzyme assay was done to check the activity of enzyme using different parameters such as pH, incubation time and temperature with change in their values at each time. The optimum working pH was 7.5 where both free and immobilized enzyme showed their maximum activity. Free enzyme showed maximum activity at 37°C while immobilized enzyme showed maximum activity between 35-40°C. . The free enzyme showed maximum activity after 30 minutes of incubation while immobilized enzyme showed maximum activity after a wide range of incubation time .

With time research on enzyme immobilization has been increased continuously. It can be concluded that in future enzyme immobilization can be a promising technique in many fields of like environmental monitoring, in biotransformations, diagnosis, pharmaceutical and food industries. Techniques which are enzyme based are

replacing chemical methods in laboratories and industries due to their efficiency, multipurpose uses and results are very fast. At present enzyme immobilization at commercial level is of low use because of high cost and storage problems of enzymes. Research in future should be focused to reduce the limitations of immobilization technique.


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