CHAPTER III

RESULTS

3.1 Partial purification of phenylalanine dehydrogenase

3.1.1 Preparation of crude extract

Crude PheDH was prepared from 10 g wet weight of *Escherichai coli* BL 21 (DE3) transformant which was cultivated in 2 liters of LB medium supplemented with 100 μg/ μl ampicillin and 0.4 mM IPTG as described in section 2.4. Crude extract contained 910 mg proteins and 5,500 units of PheDH activity. Thus, the specific activity of the enzyme in the crude preparation was 6 units/mg protein.

3.1.2 Ammonium sulfate precipitation

Crude enzyme solution was purified by ammonium sulfate precipitation in the range of 50-70% saturation. The precipitated proteins were dissolved in 10 mM potassium phosphate buffer pH 7.4 containing 0.01% (v/v) 2-mercaptoethanol, 1 mM EDTA (working buffer) and dialyzed against the same buffer. In this step, the recovered protein and enzyme activity were 290 mg and 2,450 units, respectively. The specificity activity of the enzyme was 8.4 units/mg protein. The enzyme was purified 1.4 fold with 44.4 % recovery compared with the crude extract.

3.1.3 DEAE-Toyopearl column chromatography

The enzyme from section 3.1.2 was loaded onto DEAE-Toyopearl column as described in section 2.7.3.2. The chromatographic profile is shown in **Figure 3.1**. The unbound proteins were eluted from DEAE-Toyopearl column with working buffer until the absorbance at 280 nm of eluant decreased to nearly zero. The bound

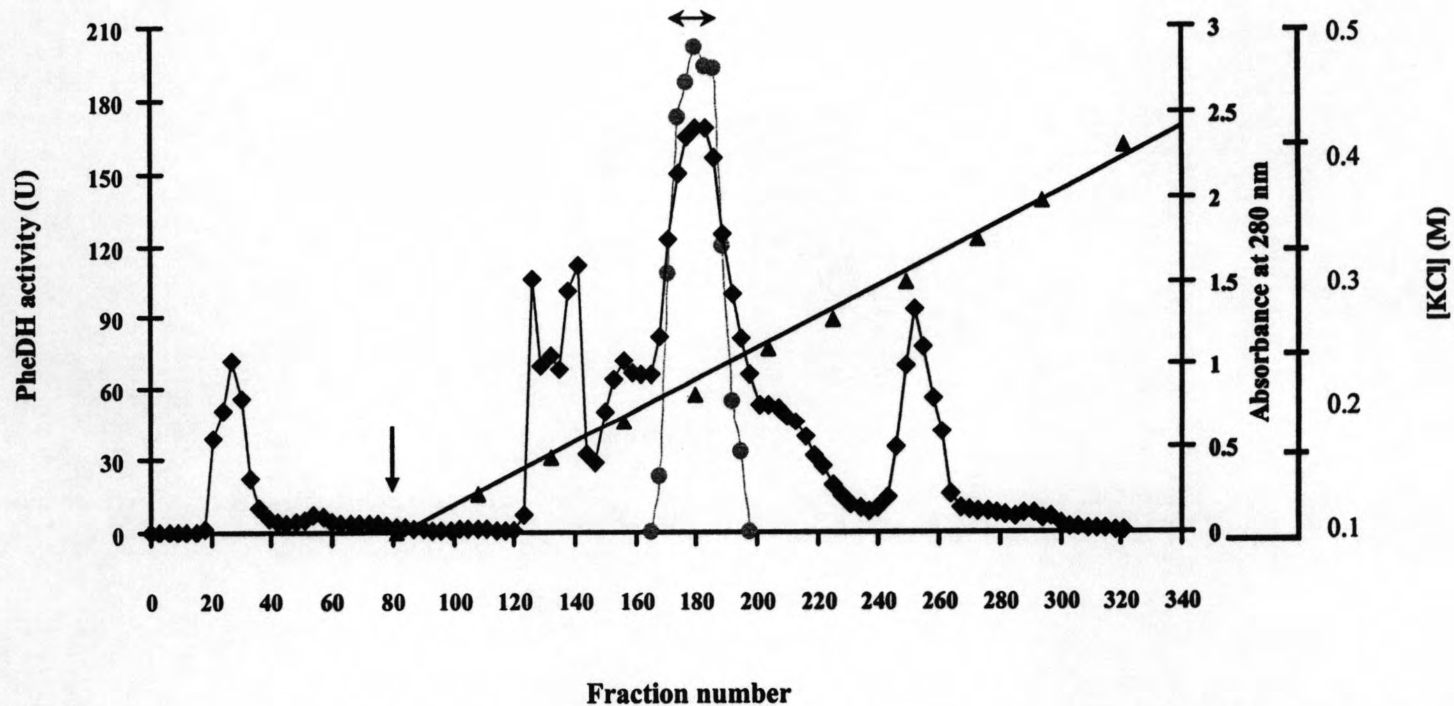


Figure 3.1 Purification of phenylalanine dehydrogenase from *E. coli* BL 21(DE3) by DEAE - Toyopearl column

The enzyme solution was applied to DEAE -Toyopearl column and washed with 10 mM potassium phosphate buffer, pH 7.4 containing 0.01 %(v/v) β-mercaptoethanol and 1 mM EDTA until A280 decreased to base line. The bound proteins were eluted by 0 - 0.5 M KCl in the same buffer at the flow rate of 1 ml/min. The fractions of 3 ml were collected. The arrow indicates where gradient started. The protein peak from fraction number 177 to 186 was pooled (⟷), ♦ A280, ● PheDH activity, — [KCl].

proteins were then eluted by linear salt gradient of 0 to 0.5 M potassium chloride in the same buffer. PheDH was eluted at around 0.2 M potassium chloride (as indicated in the profile). PheDH fractions were pooled, dialyzed against working buffer and concentrated by centricon to reduce enzyme volume. The protein from this step was 72.2 mg with 1,110 activity units of enzyme and specificity activity of the enzyme was 15.4 units/mg protein. The enzyme was purified 2.6 fold with 20.2% recovery as shown in **Table 3.1**.

3.1.4 Determination of enzyme purity and protein pattern on non-denaturing polyacrylamide gel electrophoresis and SDS-polyacrylamide gel electrophoresis

The enzyme from each step of purification was examined for its purity and protein pattern by polyacrylamide gel electrophoresis as described in section 2.7.3.3.1 and 2.7.3.3.2, respectively. The results are shown in **Figure 3.2**. The enzyme purity increased in each step of purification as the band of other proteins decreased (gel A, lane 1-3). The enzyme in lane 3 showed a few protein bands with one major band. The major band was believed to be PheDH since it moved to the same distance as that of PheDH developed by activity staining (gel B, lane 1-3). The enzyme from final step of purification still had a few contaminants (**Figure 3.3**). The molecular weight of PheDH subunit was calculated to be 44.5 kDa (**Figure 3.4**). This partial purified PheDH was then used as a source of enzyme for immobilization.

Table 3.1 Purification of recombinant phenylalanine dehydrogenase from *E. coli* BL 21(DE3)

Purification steps	Total activity (unit)	Total protein (mg)	Specific activity (units/mg protein)	% Recovery	Purification fold
Crude enzyme	5,500	910	6	100	1
40-50% saturated ammonium sulfate precipitation	2,450	290	8.4	44.4	1.4
DEAE-Toyopearl column	1,110	72.2	15.4	20.2	2.6

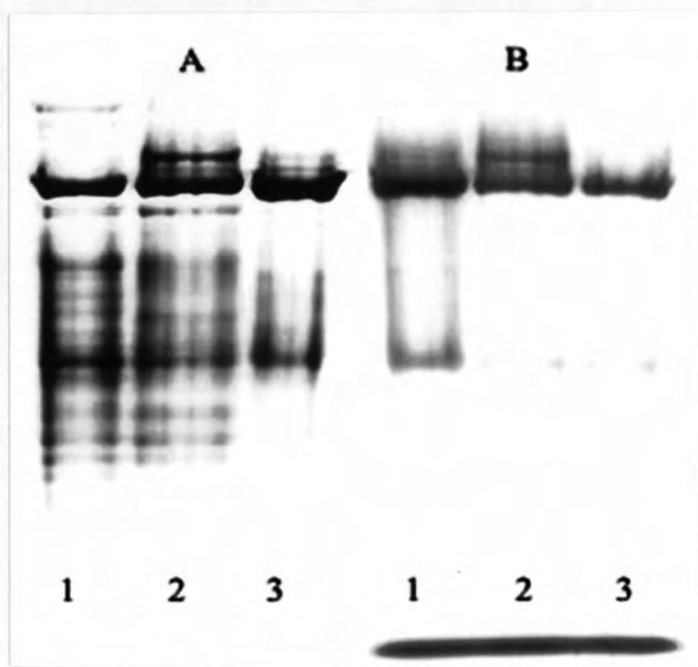


Figure 3.2 Non-denaturing PAGE of recombinant phenylalanine dehydrogenase expressed in *E. coli.*

Each lane presents each step of purification.

A. Protein staining

Lane 1	crude extract	24 μg protein
Lane 2	50-70% saturated ammonium sulfate precipitation	23 μg protein
Lane 3	DEAE-Toyopearl column	25 μg protein

B. Activity staining

Lane 1 crude extract

Lane 2 50-70% saturated ammonium sulfate precipitation

Lane 3 DEAE-Toyopearl column

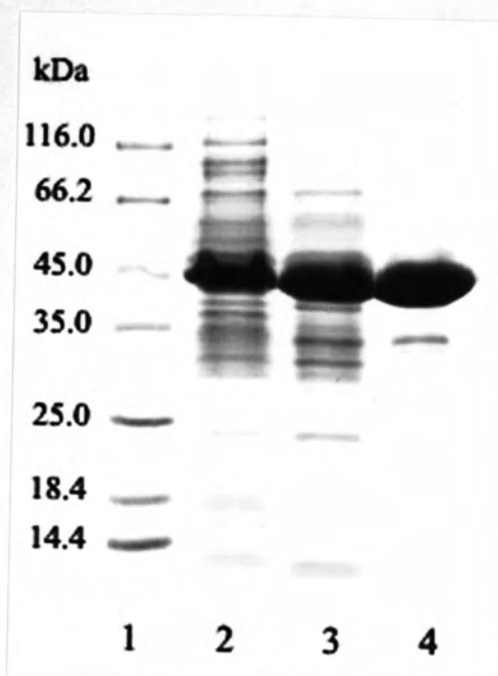


Figure 3.3 SDS polyacrylamide gel electrophoresis of phenylalanine dehydrogenase from recombinant *E. coli.*

Lane 1	= protein marker	
Lane 2	= crude extract	24 μg protein
Lane 3	= 50-70% saturated ammonium sulfate precipitation	23 μg protein
Lane 4	= DEAE-Toyopearl column	25 μg protein

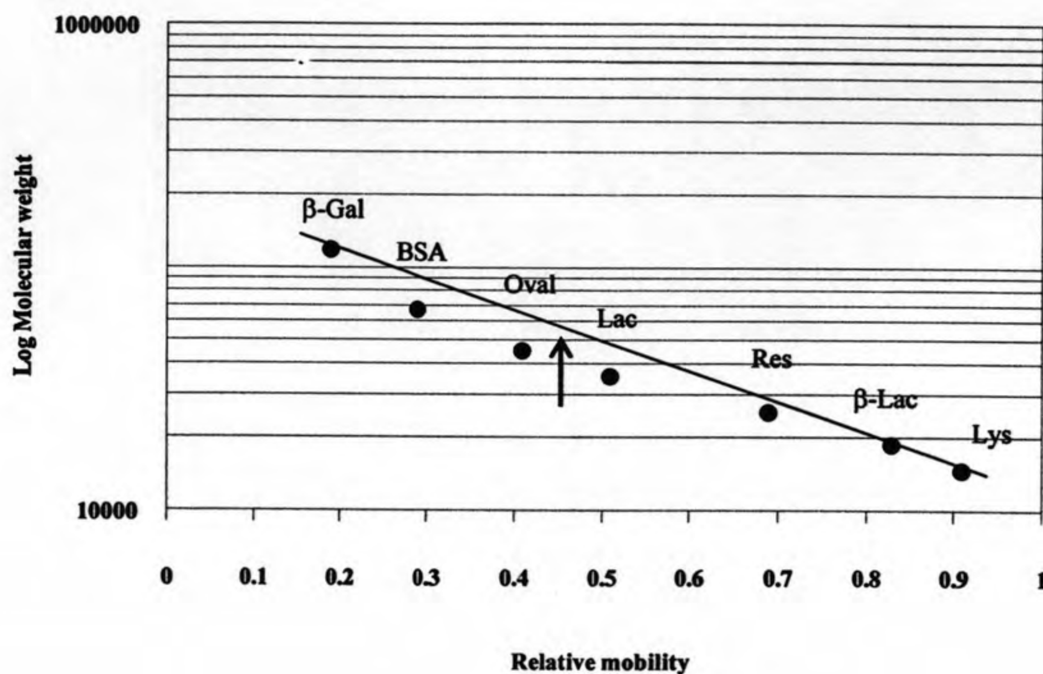


Figure 3.4 Calibation curve for molecular weight of phenylalanine dehydrogenase subunit from recombinant *E. coli* on SDS polyacrylamide gel electrophoresis.

β-Gal = β-galactosidase MW 116,000

BSA = bovine serum albumin, MW 66,200

Oval = ovalbumin, MW 45,000

Lac = lactate dehydrogenase, MW 35,000

Res = restriction endronuclease, MW 25,000

β-Lac = β-lactoglobulin, MW 18,400

Lys = lysozyme, MW 14,400 Da

Arrow indicated a determined molecular weight of PheDH

3.2 Effect of group-specific reagents on phenylalanine dehydrogenase

According to the study by Chumphukam (2004), PheDH was immobilized only via its amino groups although various methods of immobilization were employed. The immobilized PheDH exhibited good storage stability and was able to produce various amino acids from their keto substrates. Nevertheless, the activity of the immobilized enzyme was only 1.05% of its original activity. In addition, since covalent binding involves the formation of a stable covalent bond between the support and an enzyme, suitable functional groups of the enzyme should be determined. Therefore, amino acid residues which are essential for function or structure of the enzyme were elucidated by using chemical modification.

PheDH was chemically modified with a series of group-specific reagent to identify essential amino acid residues by incubating the purified enzyme with 10 mM of each modifying regent at 30°C for 20 minutes under the selective condition for each reagent as described in section 2.8. The residual activity for oxidative deamination and reductive amination were then determined and compared with those of untreated enzymes as shown in **Table 3.2**. PheDH activity was completely inhibited by 10 mM of NBS, CT, DEPC and TNBS in both of oxidative deamination and reductive amination. Hence, tryptophan, methionine, histidine and lysine residues may play an important role in the active site of the enzyme. The great loss of activity was observed when enzyme was modified with 10 mM PMSF which was specific for serine. In contrast, NAI, EDC and DTT, the modifiers of tyrosine, aspartic or glutamic and cysteine, respectively, had little effect on the enzyme activity since almost all activities retained after incubation with these reagents.

Table 3.2 Effect of various group-specific reagents on phenylalanine dehydrogenase from *E. coli* BL 21 (DE3) [a]

Group-specific reagent (10 mM)	Amino acid involed	Residual activity (%)	
		Oxidative deamination	Reductive amination
None	-	100	100
N- Acetylimidazole (NAI)	Tyrosine	100	100
N-Bromosuccinimide (NBS)	Tryptophan	0	0
N-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC)	Aspartic & Glutamic	88	94
Chloramine T (CT)	Methionine	0	0
Diethylpyrocarbonate (DEPC)	Histidine	0	0
Dithiothreitol (DTT)	Cysteine	92	87
Phenylmethylsulfonyl fluoride (PMSF)	Serine	15	43
2,4,6-Trinitrobenzene sulfonic acid (TNBS)	Lysine	0	0

[a] The enzyme was treated at 30°C for 20 minutes under the condition of pH specified in the respective modifiers. The residual activity was then assayed.

3.3 Selection of a suitable solid support and an appropriate method of surface activation

The partial purified enzyme was immobilized on several solid supports with a variety of activation techniques as described in section 2.9.2. Different immobilization methods were used for the immobilization of PheDH, i.e. covalent immobilization via its amino groups, carboxylic groups and ionic interaction. From the results obtained above (section 3.2), lysine residue is an important residue involved in enzyme catalytic activity. Therefore, to achieve maximum immobilization yield, immobilization through its carboxylic groups and immobilization via ionic interaction were also studied. All immobilized procedures were prepared under different reaction conditions such as incubation time and the activated support. Enzyme coupling was carried out by incubating18 units of PheDH with 500 mg of activated support. The enzyme activity was measured by the method described in section 2.10.

3.3.1 Immobilization via its amino groups

PheDH was immobilized by covalent linking. The immobilization of the enzyme is preformed through the intermediate of double functional group reagent such as glutaraldehyde and by dint of the occurring Schiff base reaction. The schematic diagram showing an approach used for the immobilization of PheDH is shown in **Figure 3.5**. In this form, the primary amino groups of the enzyme and the support will be attached with one molecule of glutaraldehyde (Blasi *et al.*, 2005).

3.3.1.1 γ-Aminopropyltriethoxysilane (APTS)

In previous study (Chumphukam, 2004), PheDH was successfully immobilized on silica using glutaraldehyde as a coupling agent. Therefore, to confirm the results, the optimum conditions for PheDH immobilization were re-examined.

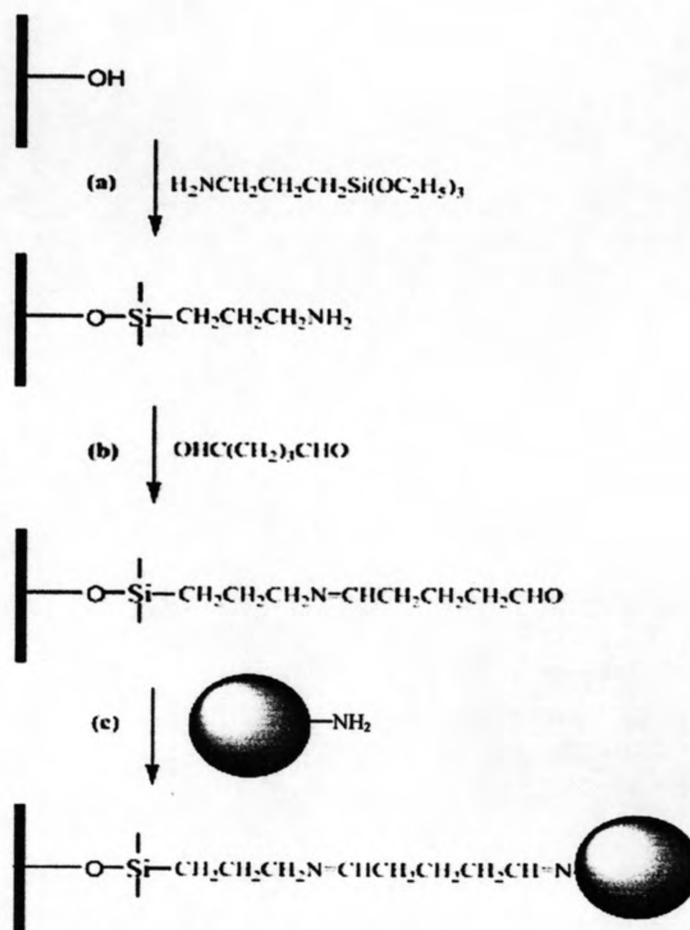


Figure 3.5 Schematic diagram showing an approach of covalent attachment of PheDH to inorganic support using glutaraldehyde as s crosslinking agent (Blasi *et al.*, 2005).

(a) silanization of a cleaned silica with γ-aminopropyltriethoxysilane (APTS)

(b) reaction of alkylamine-derivatized silica with glutaraldehyde

(c) incubation of silica with PheDH in phosphate buffer solution

3.3.1.1.1 Effect of APTS concentration

The effect of silanization procedures on the activating of a silica surface with APTS was studied. The support was treated with different concentration of APTS varying from 1.0 to 10% (v/v). These silanized supports were cross-linked with 1% (v/v) of glutaraldehyde and was incubated with the enzyme as mentioned previously (2.9.3.1A). As can be seen in **Figure 3.6**, the PheDH immobilized on silica coated with different amount of APTS had similar expressed activities. Increasing APTS concentration did not have any effect on the immobilized enzyme activity. At 1% (v/v) of APTS, the activity of PheDH immobilized exhibited the maximum of 0.126 U/ g support. Therefore, the suitable APTS concentration for silanization of silica was 1% (v/v).

3.3.1.1.2 Effect of glutaraldehyde concentration

To increase the immobilized enzyme activity, the effect of glutaraldehyde concentration on the activity of the immobilized enzyme was studied by varying glutaraldehyde concentration. After silica was silinized with 1% (v/v) of APTS, the support was then activated with different amount of glutaraldehyde in the range of 0.1 to 2.5% (v/v), in 0.1 M phosphate buffer, pH 7.0 for 2 hours at room temperature. Then 16 U of PheDH was added and incubated for 6 hours at 4°C. The result is shown in **Figure 3.7**. It showed that the immobilized enzyme possessed higher activity when 0.25% (v/v) glutaraldehyde was used. The activity of the immobilized enzyme decreased when the immobilized PheDH was activated at high concentration of glutaraldehyde. Consequently, glutaraldehyde concentration at 0.25% (v/v) was selected for activation of solid support prior to enzyme immobilization.

3.3.1.1.3 Effect of coupling time

The effect of the coupling time on the activity of immobilized enzyme is shown in **Figure 3.8**. The coupling time was varied from 0.5 to 12 hours at 4°C. The

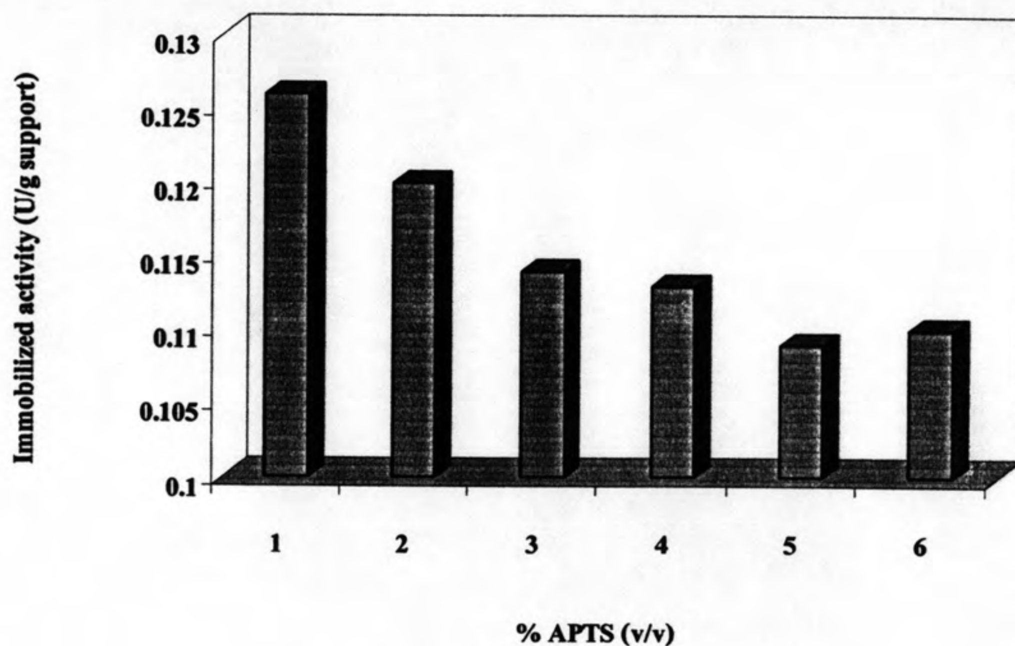


Figure 3.6 Influence of APTS concentrations on the amount of PheDH immobilization on silica. The silica (500 mg) was silanized with different concentration of APTS, activated with 1% (v/v) glutaraldehyde and incubated with 16 U of enzyme at 4°C for 6 hours. Results shown were average values of duplicate experiments.

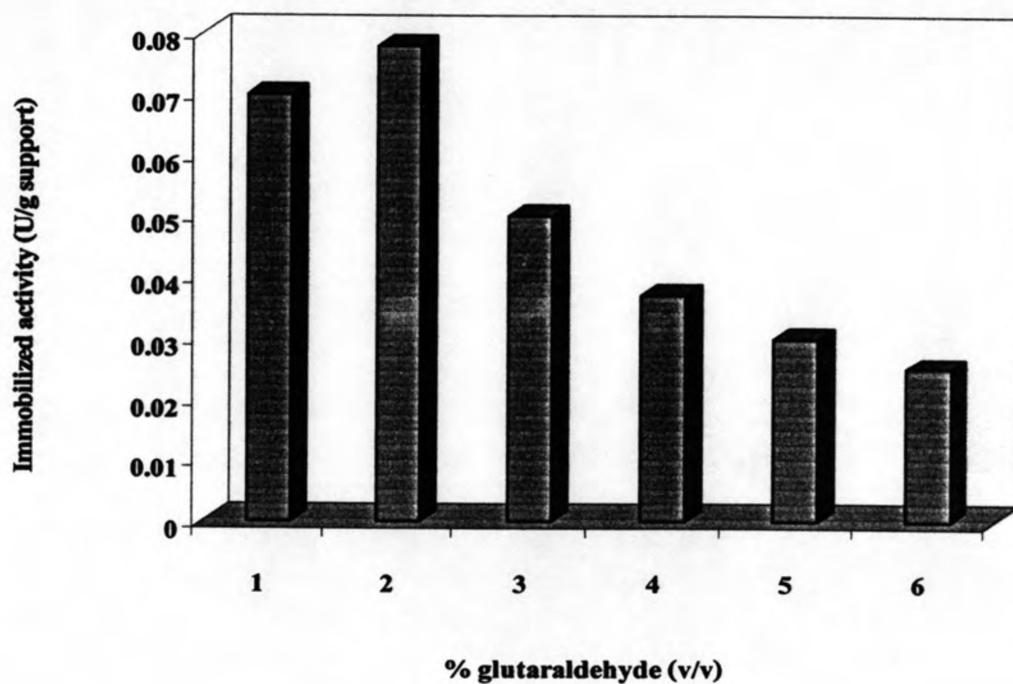


Figure 3.7 Influence of glutaraldehyde concentrations on the PheDH immobilization on silica. Silica (500 mg) was silanized with 1% (v/v) APTS, activated with different concentration of glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) and incubated with 16 U of enzyme at 4°C for 6 hours. Results shown were average values of duplicate experiments.

results showed that the maximum immobilized activity increased with increasing coupling time up to 8 hours (0.09 U/g support). Prolonging of the coupling time did not increase the immobilized activity. Therefore, an incubation time of 8 hours was used for the next experiment.

3.3.1.1.4 Effect of enzyme concentration

In order to obtain higher efficiency of immobilization, influences of enzyme concentration was studied. **Figure 3.9** shows the immobilized activity during the process of attachment of the enzyme to the support. Different amount of PheDH applied to silica were varied from 2 to 40 U/ 500 mg support. It can be observed that the immobilized activity initially increased with increasing enzyme added to the support. On the other hand, at enzyme concentration higher than 18 units, the immobilized activity remained constant. Therefore, the optimal amount of PheDH used for immobilization was 18 units with 0.102 U/g support of immobilized activity.

The summary of optimal conditions for covalent immobilization of PheDH using glutaraldehyde as a coupling agent is shown in **Figure 3.10**. Under these optimum conditions, the activity of the immobilized enzyme was 0.102 U/g support.

3.3.1.2 1,6-Diaminohexane

PheDH was covalently immobilized onto aminated alumina and aminated chitosan and crosslinking with glutaraldehyde. The aminated support was modified from epoxy alumina and epoxy chitosan which was converted into amino group with 1,6-diaminohexane as described in section 2.9.2.2B (Yakub Arica *et al.*, 2003; Bayramoglu *et al.*, 2005). Silica was not a good support activated with this method because it dissolved when incubated with 1, 4-butanedioldiglycidyl ether solution. The schematic diagram is shown in **Figure 3.11**. 1,4-Butanediol diglycidyl ether was used to give an epoxy-functional group to the support. One of the

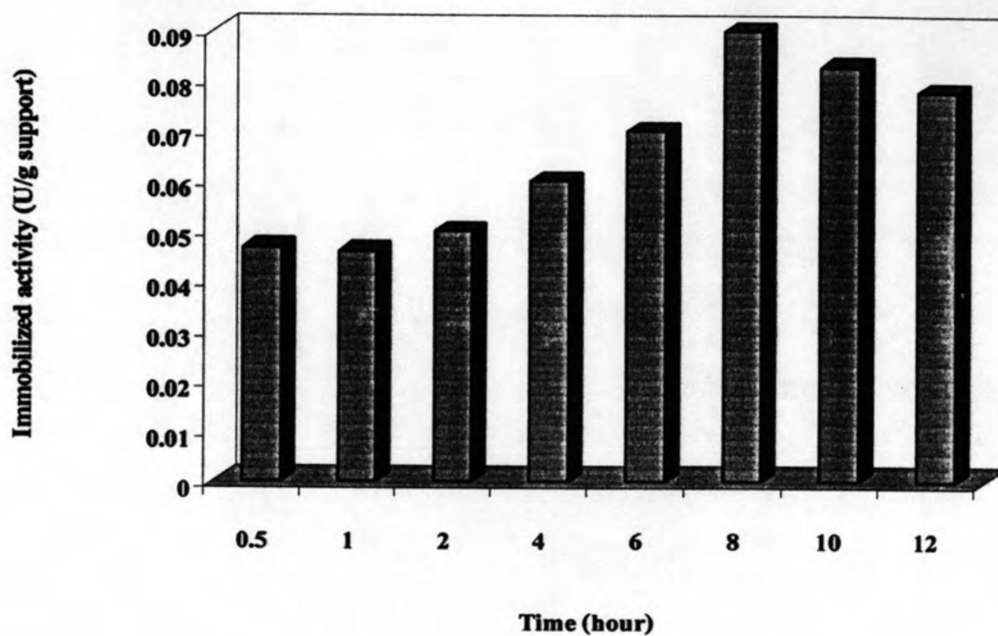


Figure 3.8 Influence of coupling time on the amount of PheDH immobilization on silica. The silica (500 mg) was silanized with 1% (v/v) APTS, activated with 0.25% (v/v) glutaraldehyde and incubated with 16 U of enzyme at 4°C at different incubation times. Results shown were average values of duplicate experiments.

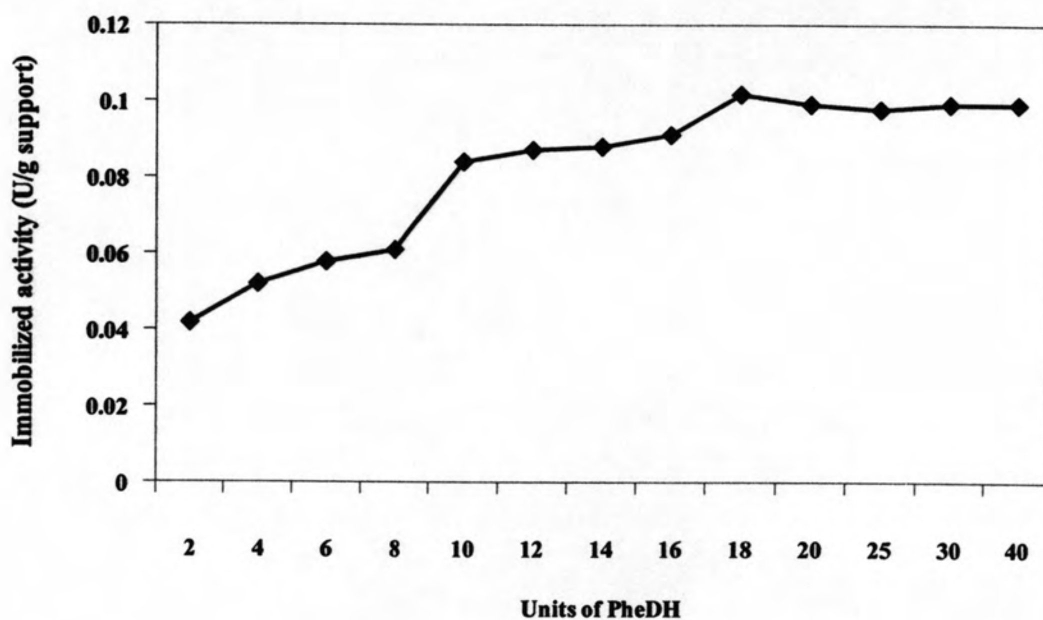


Figure 3.9 Influence of the amount of PheDH applied on the exhibited activity. The silica (500 mg) was silanized with 1% (v/v) APTS, activated with 0.25% (v/v) glutaraldehyde and incubated with PheDH solution (2–40 U) at 4°C for 8 h. Results shown were average values of duplicate experiments.

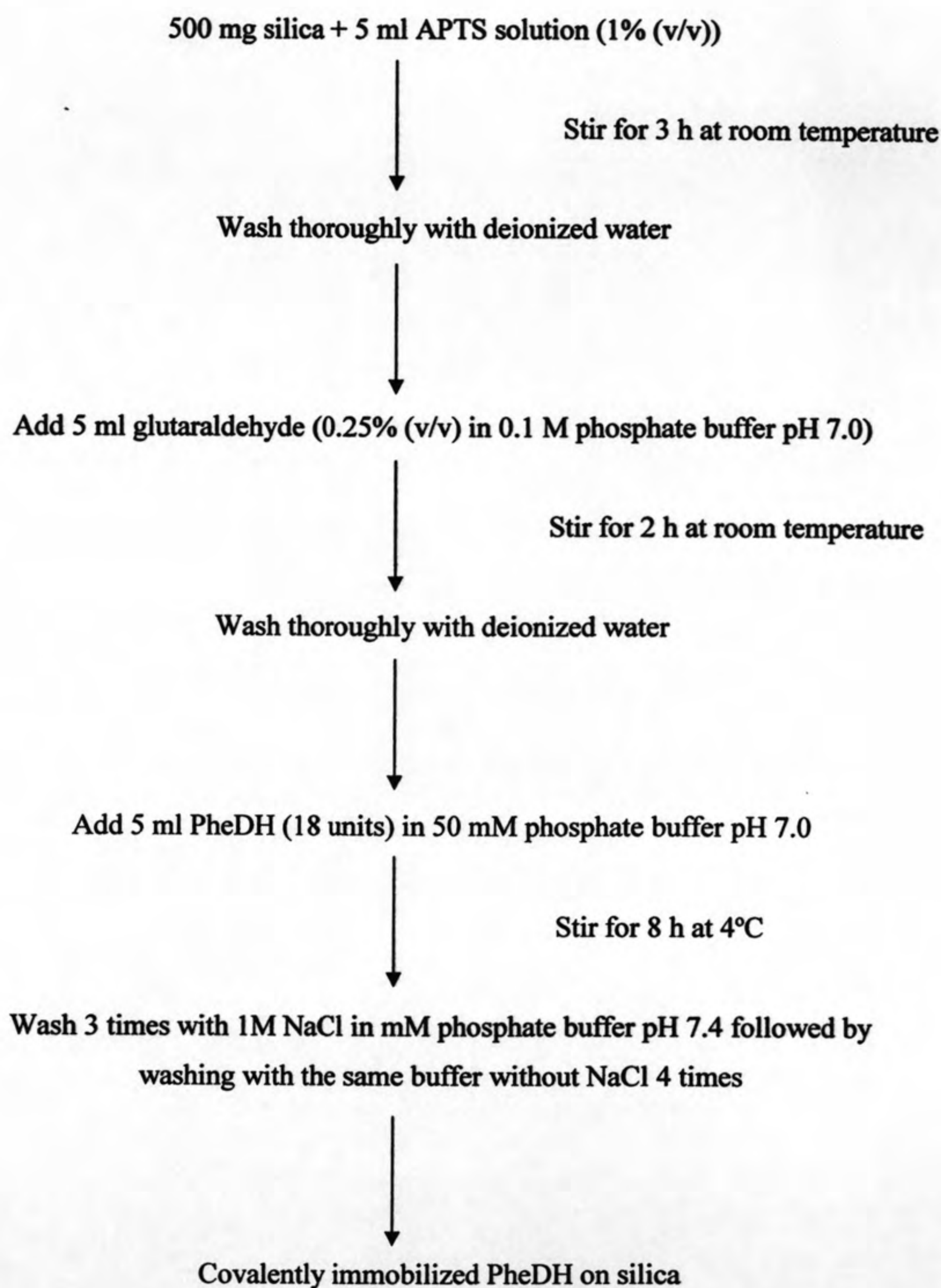


Figure 3.10 The optimal conditions for PheDH immobilization on silica by activation with APTS and crosslinking with glutaraldehyde.

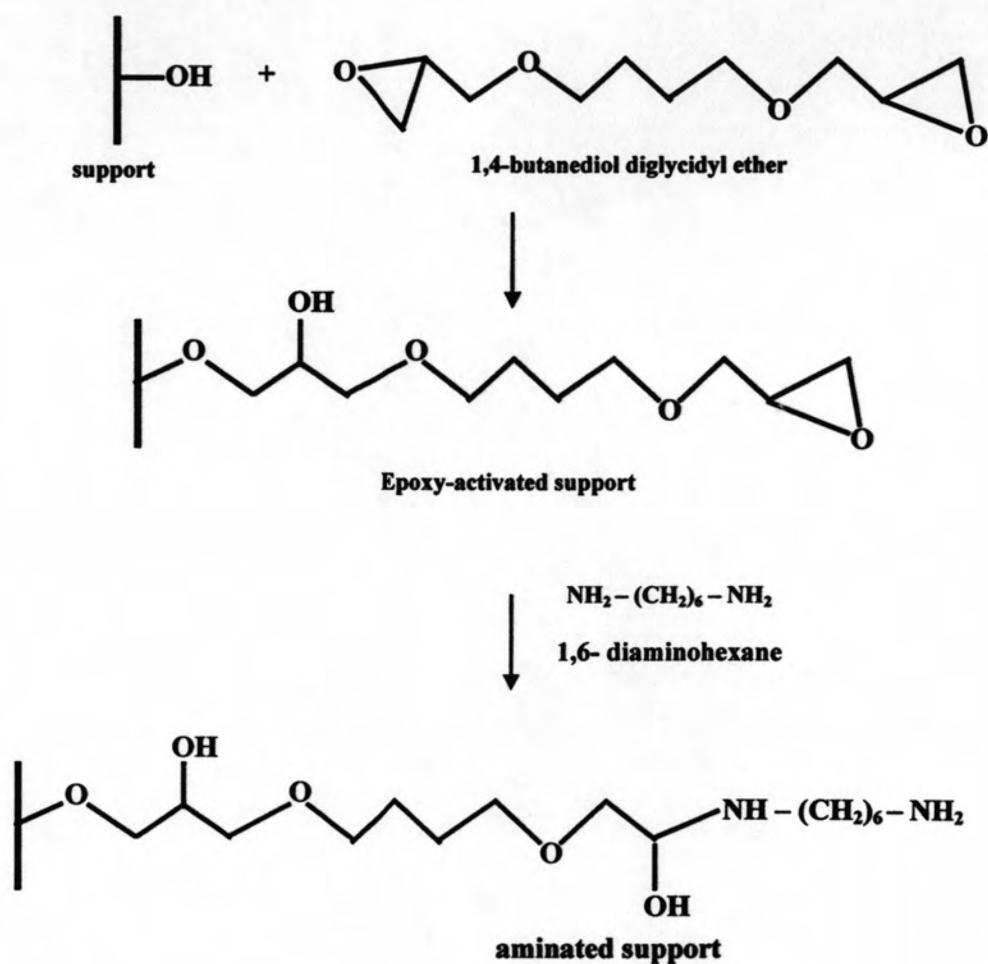


Figure 3.11 Schematic diagram showing the preparation of epoxy activated support using 1,4-butanedioldiglycidyl ether which was converted into amino-group with 1,6-diaminohexane (Ragnitz *et al.*, 2001; Yakub Arica *et al.*, 2003).

oxirane group of the support was converted to amino group by reacting with 1,6-diaminohexane. **Figure 3.12** shows the PheDH immobilized on various aminated supports (alumina and chitosan). The immobilization on both aminated supports showed very low immobilized activity, since only 0.094 U/g support was bound to epoxy-chitosan and 0.035 U/g support for epoxy alumina. Thus, this method was not appropriate for the immobilization of PheDH.

3.3.2 Immobilization via ionic interaction

Polyethylenemine (PEI) is one of polycations reported to have a positive effect on enzyme activity and stability. The polymer had been shown to be effective for immobilization of biocatalysts to solid support by adsorption (Atia, 2005). PEI was grafted onto the surfaces of carrier and the composite support was formed (**Figure 3.13**). The protonated PEI macromolecule will produce strong physical adsorption towards the negatively charged enzyme protein (Gao *et al.*, 2006)

In this study, PheDH was immobilized via ionic adsorption and crosslinking the adsorbed PheDH with glutaraldehyde on various ionic supports including silica, alumina, chitosan and epoxy-alumina as shown in **Figure 3.14.** Epoxy-chitosan was not an appropriate support because chitosan beads were dissolved and broken when they were activated with PEI. Only the enzyme immobilized on chitosan and epoxy-alumina via ionic adsorption was found to be maximal with the immobilized activity of 0.085 and 0.089 U/g support, respectively. When ionically adsorbed PheDH was treated with glutaraldehyde as described in section 2.9.3.2B, the immobilized activity was lower in all cases expect immobilization on silica. The immobilized enzyme via ionic interaction showed much lower efficiency than APTS method.

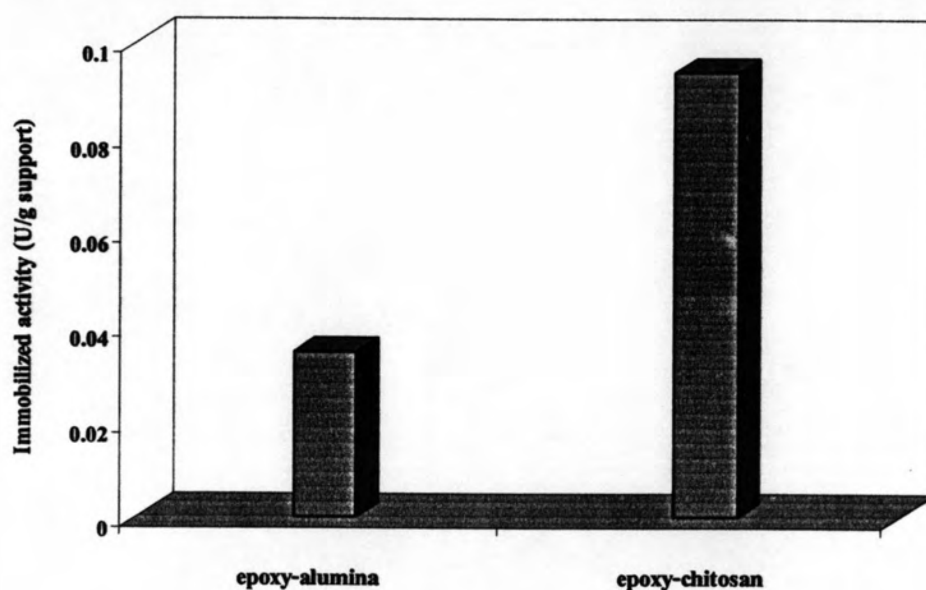


Figure 3.12 Immobilization of phenylalanine dehydrogenase by 1,6-diaminohexane method. Five hundred milligrams of epoxy support was aminated with 1% (w/v) 1,6-diaminohexane, reacted with 1% (v/v) glutaraldehyde and incubated with 18 units of PheDH at 4°C for 6 h. Results shown were average values of duplicate experiments.

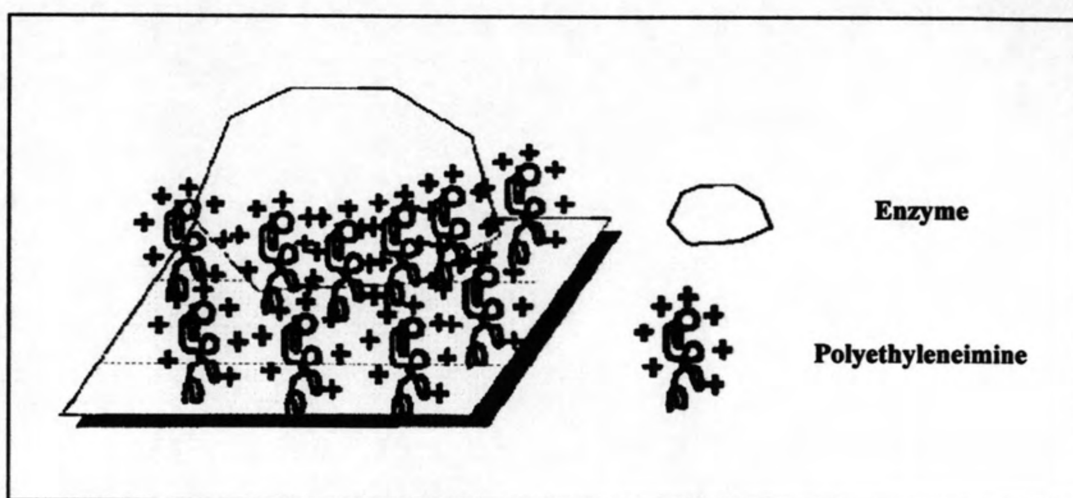


Figure 3.13 Immobilization of phenylalanine dehydrogenase via ionic interaction by using polyethyleneimime (Mateo *et al.*, 1999).

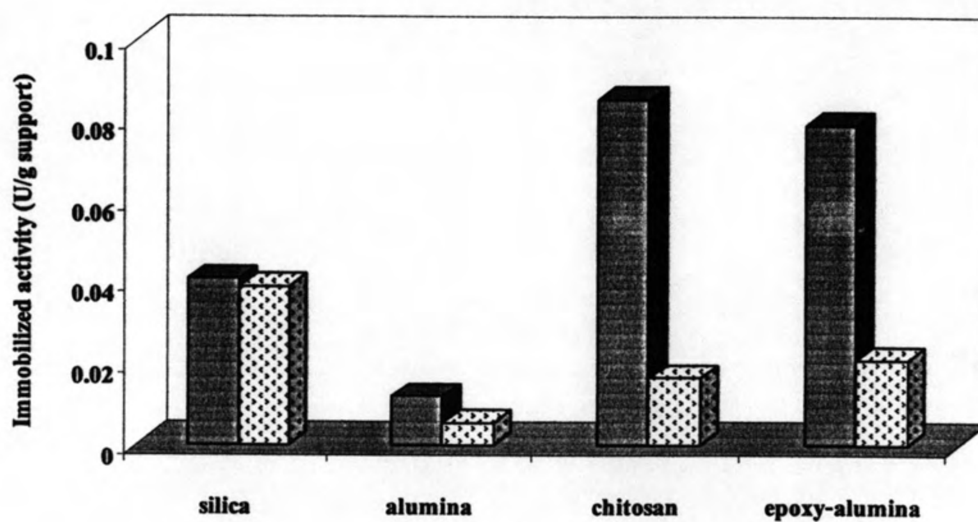


Figure 3.14 Immobilization of phenylalanine dehydrogenase by polyethyleneimine (PEI) method. Adsorbed on ionic support (■), cross-linked with glutaraldehyde (⊡)

3.3.3 Immobilization via carboxyl group of enzyme

The activation of carboxyl group of the surface PheDH was achieved by reacting with carbodiimide (*N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride, EDC). The covalent coupling proceeds by two steps (**Figure 3.15**): first, EDC reacted with carboxyl groups on the enzyme surface to form an active *O*-acrylisourea intermediate. Secondly, it was allowed to couple to the amino groups of the support (aminated alumina, aminated silica and chitosan) forming an amide bond. The results are shown in **Figure 3.16**. It can be seen that the covalent immobilized PheDH on silica showed higher activity than that of other aminated supports. For this reason, silica was applicable for immobilization of the PheDH by covalent coupling with EDC.

The overall result of the different immobilization procedures on various supports is summarized in **Figure 3.17** (only the highest immobilized activity from each immobilization method was shown). Among various immobilization method used, the immobilization of PheDH via its carboxylic groups gave the highest immobilized activity on silica when compared with other methods and supports. Therefore, in this study, this method was chosen for the immobilization of PheDH and the most appropriate support was silica.

3.4 Optimization of the immobilization method

A method for immobilization of PheDH on aminated silica by activating the carboxyl group of the enzyme using carbodiimide coupling agent has been successfully developed. The effects of immobilization parameters, such as activation time, APTS concentration, EDC concentration, coupling time and enzyme loading were investigated.

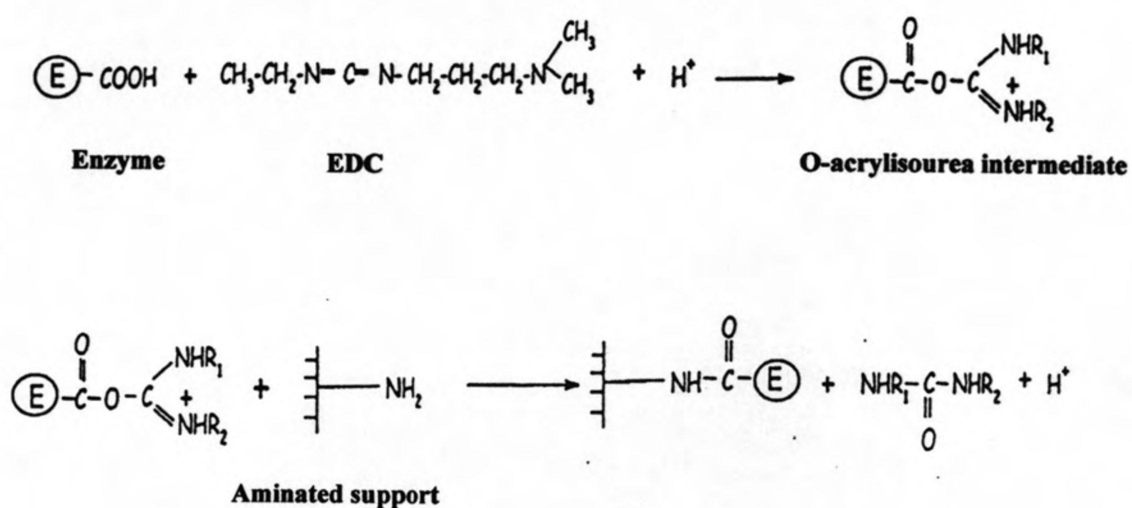


Figure 3.15 Schematic illustration of activation of carboxyl groups by EDC for the covalent immobilization of enzyme (Martin *et al.*, 2002).

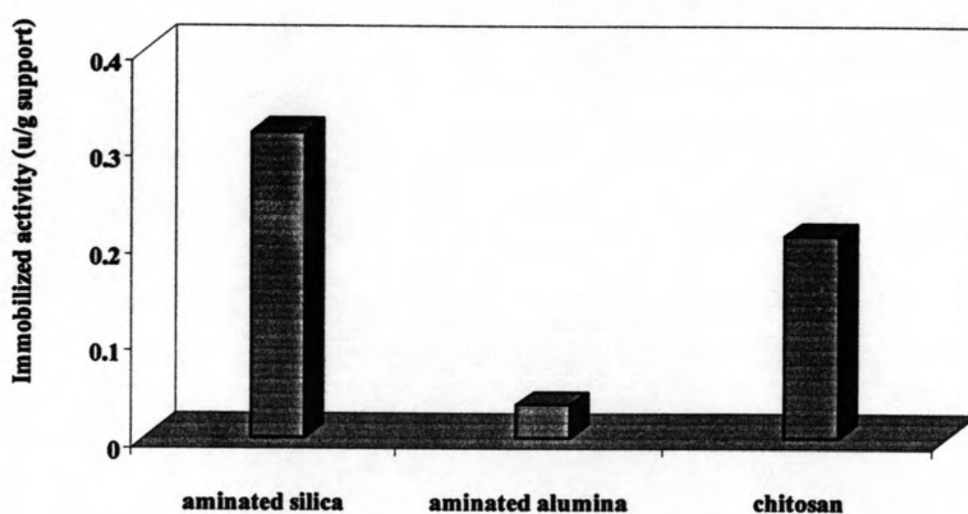


Figure 3.16 Immobilization of phenylalanine dehydrogenase by carbodiimime (EDC) method. The support 500 mg was incubated with activated PheDH (18 U) which was reacted with EDC (5 mg). The reaction was incubated at 4°C for 4 h. Results shown were average values of duplicate experiments.

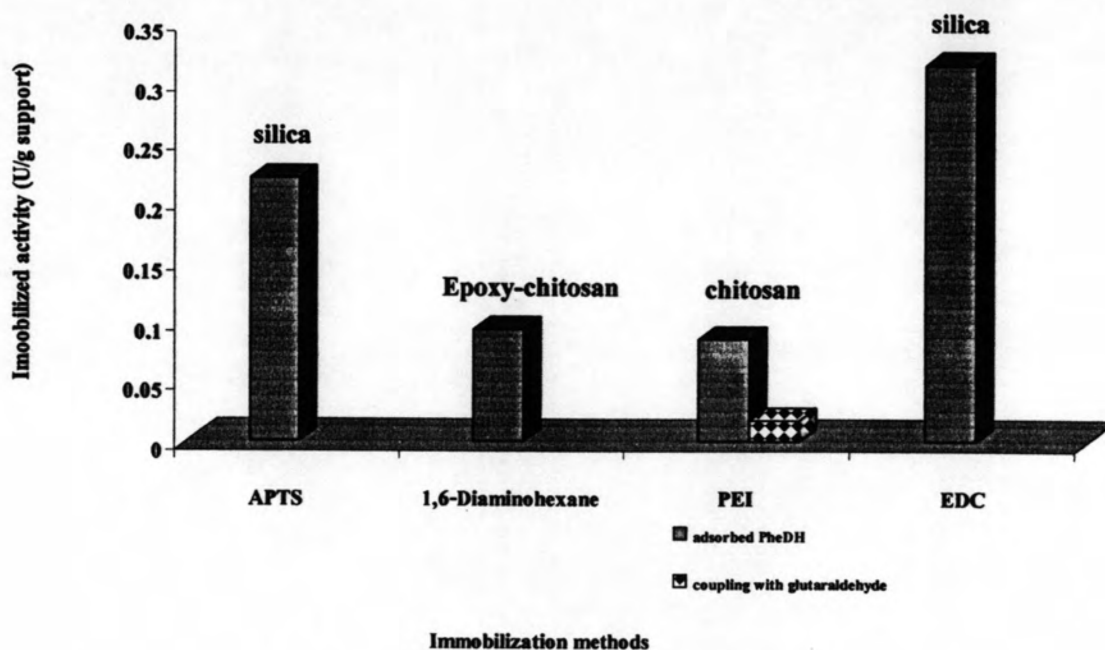


Figure 3.17 Immobilization of phenaylanine dehydrogenase on various support materials and different activation method.

APTS = γ-aminopropyltriethoxy silane

PEI = polyethyleneimine

EDC = *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride

3.4.1 Effect of activation time

The activation time between the surface carboxylic group of PheDH and water-soluble carbodiimide was investigated. The activation time was varied from 2 to 10 hours at 4°C. As presented in **Figure 3.18**, the optimal time for the activation was 6 hours which exhibited maximum immobilized activity (0.57 U/g support). When activation time was above 6 hours, the activity of immobilized enzyme moderately decreases.

3.4.2 Effect of immobilization time

The relationship between the immobilized enzyme activity and immobilization time is shown in **Figure 3.19**. It can be seen that the activity of the immobilized enzyme gradually increased from 4-21 hours and was maximum at 12 hours. Prolonging the incubation time did not increase the immobilized activity. Therefore, the optimum immobilization time of 21 hours was found to be most suitable.

3.4.3 Effect of the APTS concentration

The effect of the APTS concentration used for the activation of the silica support on the immobilized enzyme activity is shown in **Figure 3.20**. The silica was treated with different concentration of APTS varying from 1.0 to 10% (v/v) before used as a carrier for PheDH immobilization. The immobilized enzyme was not very affected by the APTS concentration during treatment. The modification with high APTS concentrations presented a higher inactivating effect of the immobilized activity. Therefore, the suitable APTS concentration for silanization of silica was 2% (v/v).

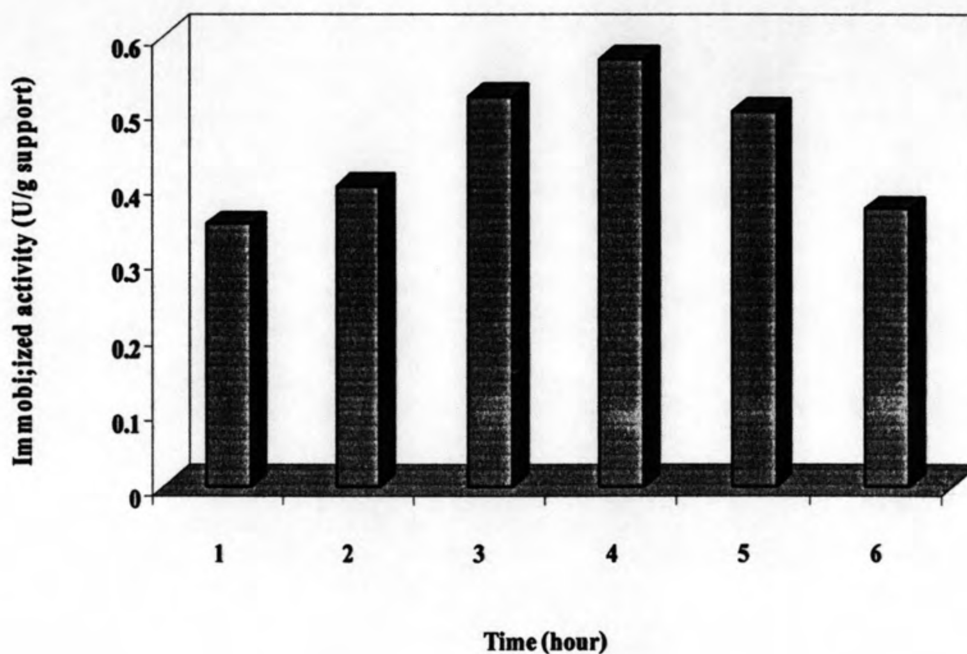


Figure 3.18 Influence of activation time on the amount of phenylalanine dehydrogenase immobilized on silica. Eighteen units of enzyme was activated with 5 mg EDC and incubated at different activation time. After five hundred milligrams of silica were activated with 1% (v/v) of APTS, they were added into EDC activated enzyme and incubated at 4°C for 12 h. Results shown were average values of duplicate experiments.

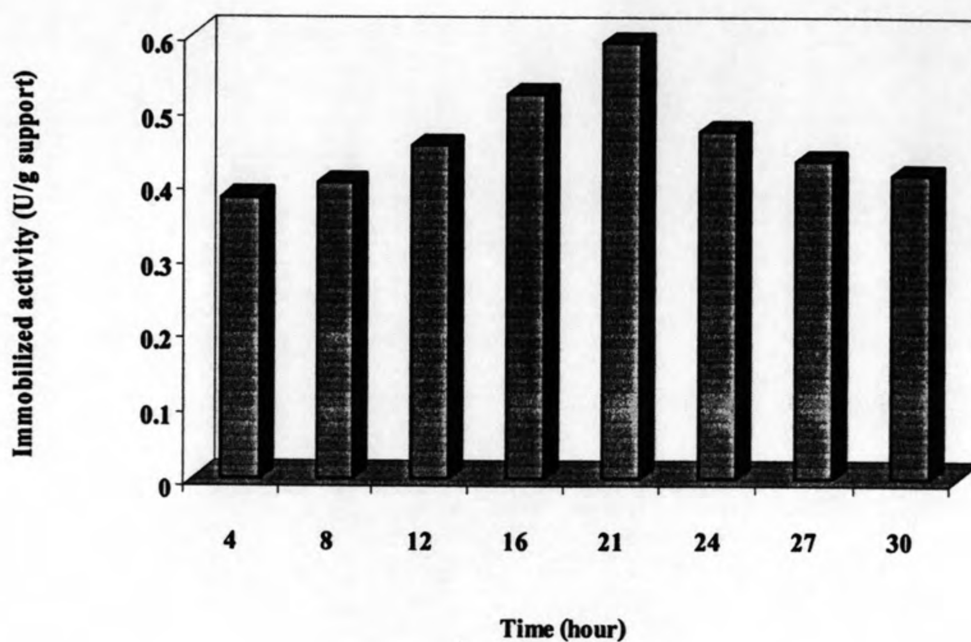


Figure 3.19 Influence of coupling time on the amount phenylalanine dehydrogenase immobilization on silica. Eighteen units of enzyme was activated with 5 mg EDC and incubated for 6 h. After five hundred milligrams of silica was silanized with 1% (v/v) of APTS, it was added into EDC activated enzyme and incubated at 4°C at different coupling time. Results shown were average values of duplicate experiments.

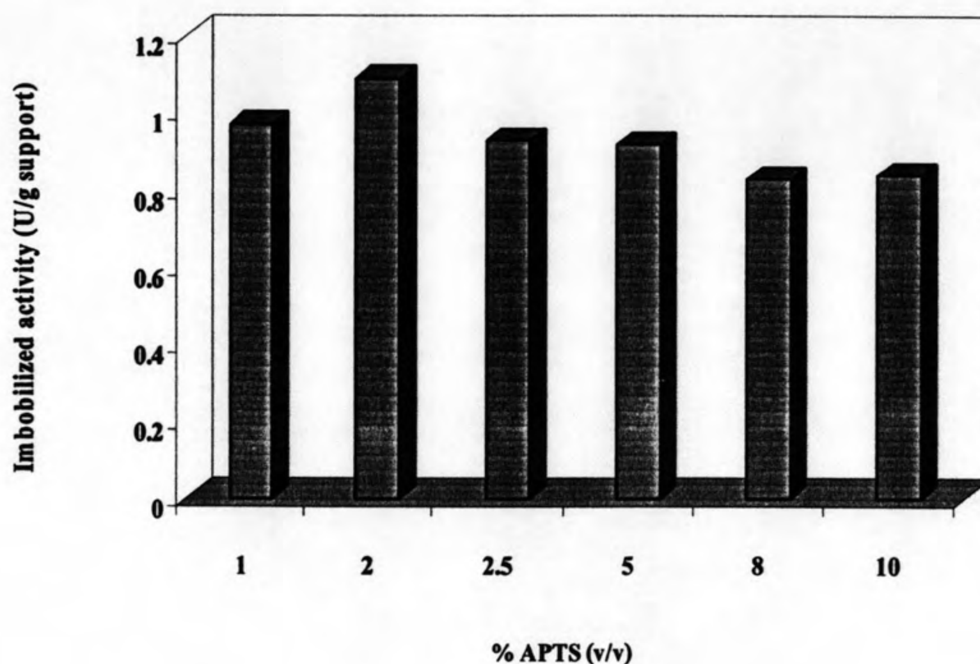


Figure 3.20 Influence of APTS concentration on the amount phenylalanine dehydrogenase immobilized on silica. Eighteen units of enzyme was activated with 5 mg EDC and incubated for 6 h. The silica (500 mg) which was silanized with different concentration of APTS was added into EDC activated enzyme and incubated at 4°C for 21 h. Results shown were average values of duplicate experiments.

3.4.4 Effect of the carbodiimide concentration

Prior to the immobilization of PheDH onto aminated silica, the surface carboxylic group of the enzyme was activated with EDC coupling agent. Thus, the effect of carbodiimide concentration on the activity of the immobilized enzyme was examined by varying EDC concentration from 1 to 100 mM. The results are presented in **Figure 3.21**. The immobilized activity was gradually decreased at higher EDC concentration. The optimum EDC concentration for PheDH immobilization was 10 mM and was used in the immobilization process in further experiments.

3.4.5 Effect of enzyme concentration

In order to achieve binding of high level of enzyme with high retention of immobilized activity, the influence of the PheDH concentration was investigated. The effect of the PheDH concentration used during the immobilization step on the immobilized activity is shown in **Figure 3.22**. Different amount of the PheDH applied to 10 mM EDC coupling agent was varied from 3 units to 35 units. The activated PheDH was further immobilized under the optimal condition (section 3.3.4) and the immobilization yield was calculated as described in section (2.12). From the result obtained, the activity increased with increasing PheDH concentration up to 25 U per 500 mg support. After that, further increase of enzyme concentration had no effect on the immobilized activity. This could be due to the saturation of the given quantity of the support. The immobilized yield was found to be inversely proportional to the immobilized activity. The optimal amount of PheDH used in the reaction was 25 U and the immobilized activity was found to be 1.41 U/g support with the immobilized yield of 5.17.

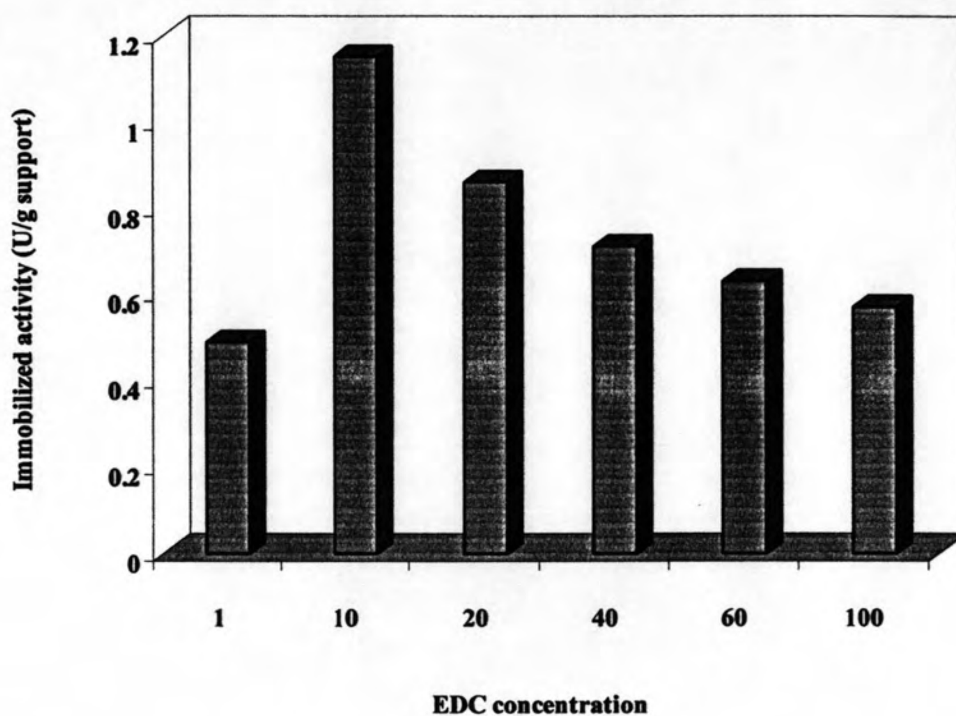


Figure 3.21 Influence of EDC concentration on the amount phenylalanine dehydrogenase immobilized on silica. Eighteen units of enzyme was activated with different EDC concentration and incubated for 6 h. After silica (500 mg) was silanized 2% (v/v) of APTS, it was added into EDC activated enzyme and incubated at 4°C for 21 h. Results shown were average values of duplicate experiments.

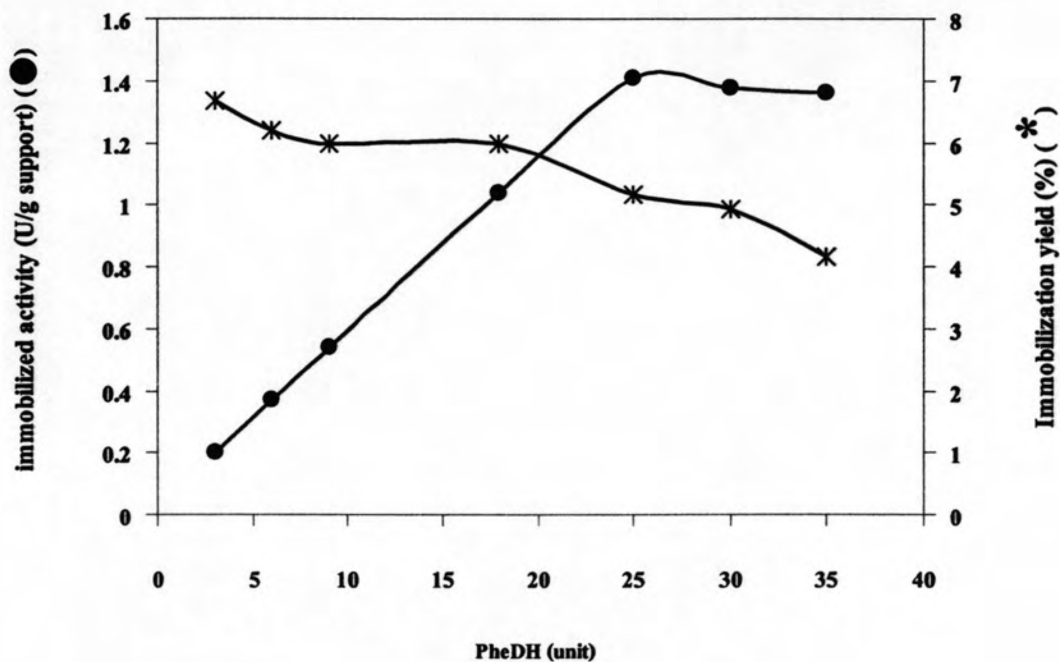


Figure 3.22 Influence of PheDH concentration on the amount phenylalanine dehydrogenase immobilization on silica. Different amount of PheDH was activated with 10 mM EDC and incubated for 6 h. After five hundred milligrams of silica was silanized with 2% (v/v) of APTS, it was added into EDC activated enzyme and incubated at 4°C for 21 h. Results shown were average values of duplicate experiments.

3.5 Characterization of the free and immobilized phenylalanine dehydrogenase

A comparative study between free and immobilized PheDH is provided in terms of pH and temperature optimum, pH and temperature stability, storage stability and reusability.

3.5.1 Effect of pH on phenylalanine dehydrogenase activity

The effect of pH on both forms of enzyme activity was examined at various pHs of buffer ranging from 8.5 to 13.0 as mentioned in section 2.13.1. The pH of each reaction mixture was measured with the pH meter after the reaction. Activity at different pH is shown in **Figure 3.23**. The pH profiles for free and immobilized enzyme activity were almost the same and both enzymes showed an optimum pH of 9.5 in the presence of glycine-KCl-KOH, pH 10.0.

3.5.2 Effect of temperature on phenylalanine dehydrogenase activity

The optimum temperatures on the activity of free and immobilized enzymes were determined by varying incubated temperatures as described in section 2.13.2. The result is shown in **Figure 3.24**. The optimum temperature (40°C) of PheDH was not altered by immobilization. The immobilized PheDH, however, demonstrated higher residual activity above 45°C as compared to that of the free PheDH.

3.5.3 Effect of pH on phenylalanine dehydrogenase stability

The pH stability of the free and immobilized enzyme was also studied as described in section 2.13.3. Both enzymes was preincubated at 30°C for 20 minutes in various 10 mM buffers at various pHs ranging from 4.0 to 13.0. The result was shown

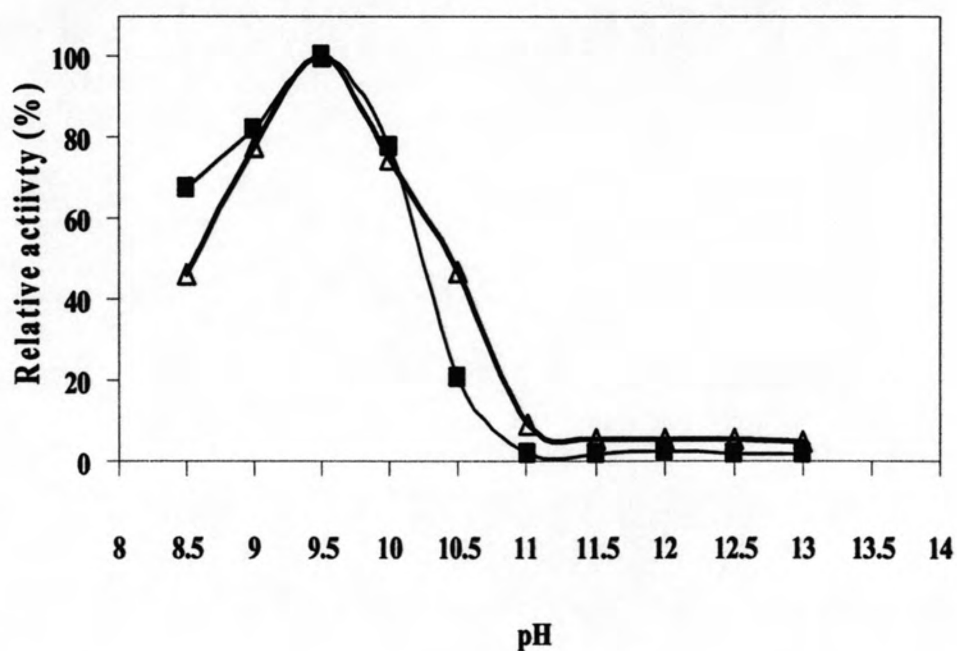


Figure 3.23 Effect of pH on the activity of free and immobilized phenylalanine dehydrogenase. The activity of free (■) and immobilized (△) PheDH were measured at different pHs with 200 mM glycine-KCl-KOH buffer (pH range 8.5-13.0) at room temperature. The highest activity was regarded as 100%. Results shown were average values of duplicate experiments.

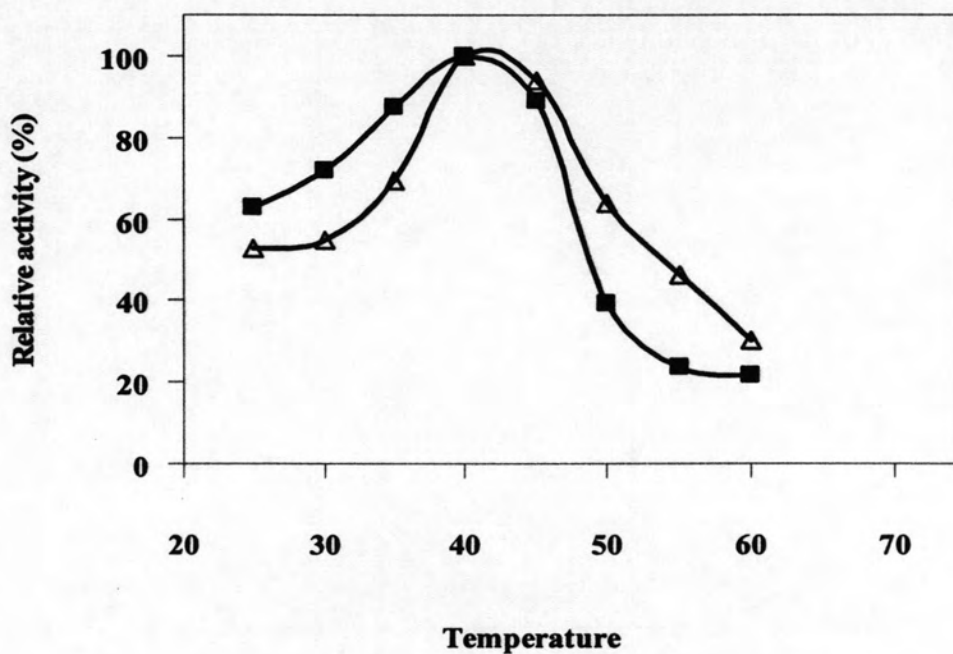


Figure 3.24 Effect of temperature on the activity of free and immobilized phenylalanine dehydrogenase. The free (■) and immobilized (△) PheDH was performed in 200 mM glycine-KCl-KOH buffer (pH 9.0) at various temperatures. Results shown were average values of duplicate experiments.

in **Figure 3.25**. The free PheDH was stable over the pH range of 5.0 to 8.5 while immobilized enzyme had a slightly broader pH range from 5.0 to 12.0.

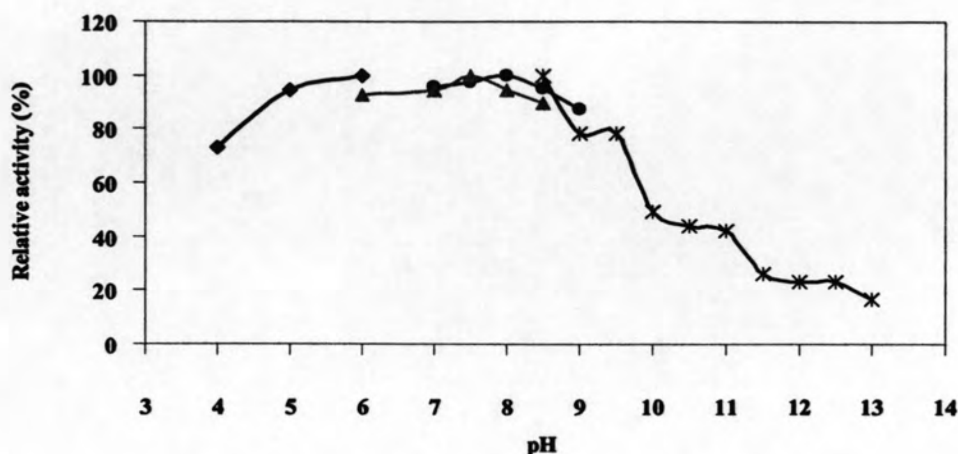
3.5.4 Effect of temperature on phenylalanine dehydrogenase stability

The thermostability of the free and immobilized was determined as described in section 2.13.4. The free and immobilized enzyme was preincubated at various temperatures ranging from 25°C to 75°C for 10 min. The residual activities were then assayed under standard condition at 25°C (section 2.10). Both forms of enzyme preparations retained its full activity at temperature up to 35°C (**Figure 3.26**). The immobilized enzyme lost about half life of its activity at 45°C whereas free enzyme retained only 20% of its activity. When incubating both enzymes at temperatures higher than 50°C, the enzyme activities rapidly declined and at 75°C, both free and immobilized PheDH completely lost their activity.

3.5.5 Storage stability

The storage stability of free and immobilized PheDH were investigated at 4°C as well as at room temperature and the results are shown in **Figure 3.27**. They were stored in 10 mM potassium phosphate buffer pH 7.4 with 0.01% (v/v) 2-mercaptoethanol and 10% (v/v) glycerol. The residual activity was measured during a 40 days storage period. It can clearly be seen that after 20 days of storage at room temperature, the residual activity of the immobilized PheDH preparation decreased slower than that of the free enzyme. The free enzyme lost all its activity within 30 days whereas the immobilized enzyme still retained its activity up to 20%. At 4°C, the residual activity of both enzymes was slightly decreased during a 40 days storage period. The free and immobilized PheDH retained their residual activities about levels of 39.71% and 33.10%, respectively.

(A)



(B)

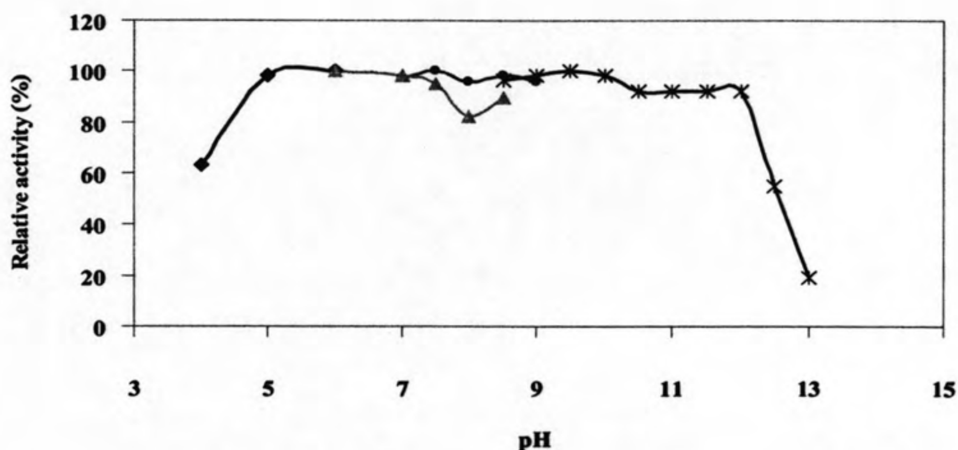


Figure 3.25 Effect of pH on the stability of free and immobilized phenylalanine dehydrogenase. Free (A) and immobilized (B) enzymes were treated in buffers at various pH's. The incubation was carried out 30°C for 20 minutes and the residual activities were assayed under standard condition. The 10 mM buffer used were citrate buffer (pH 4.0-6.0; ■), potassium phosphate buffer (pH 6.0-7.5; ▲), Tris-HCl buffer (pH 7.0- 9.0; ●) and glycine-KCl-KOH buffer (pH 8.5-13.0; ✳). Results shown were average values of duplicate experiments.

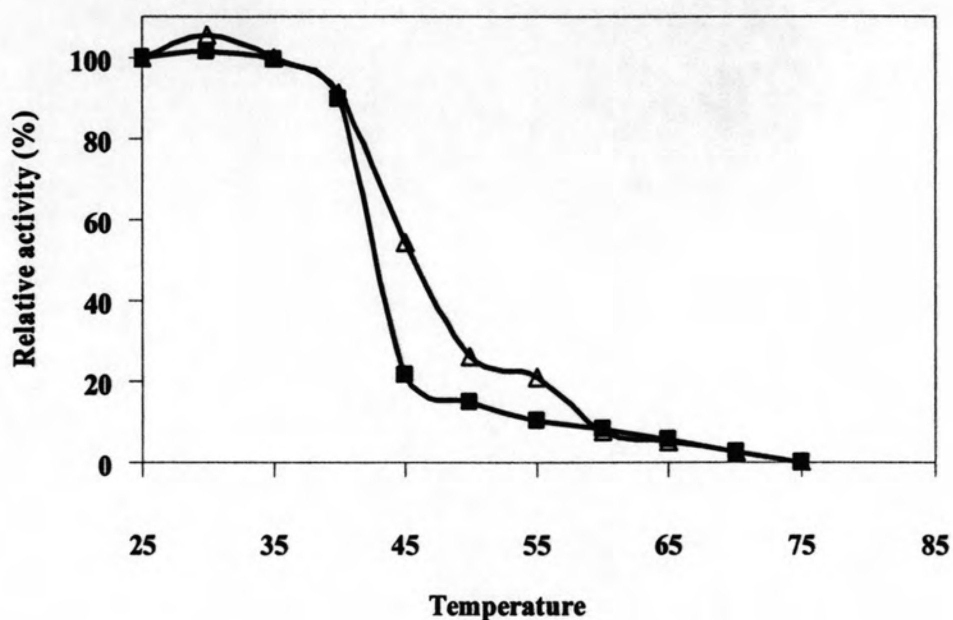
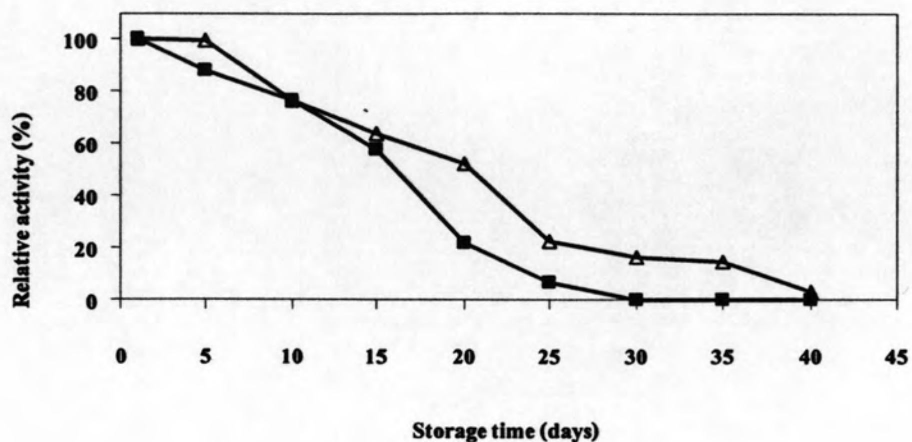


Figure 3.26 Effect of temperature on the stability of free and immobilized phenylalanine dehydrogenase. Free (■) and immobilized (△) enzymes were preincubated in 10 mM phosphate buffer, pH 7.4 for10 minutes at various temperatures and then were assayed for residual activities at room temperature. Results shown were average values of duplicate experiments.

(A)



(B)

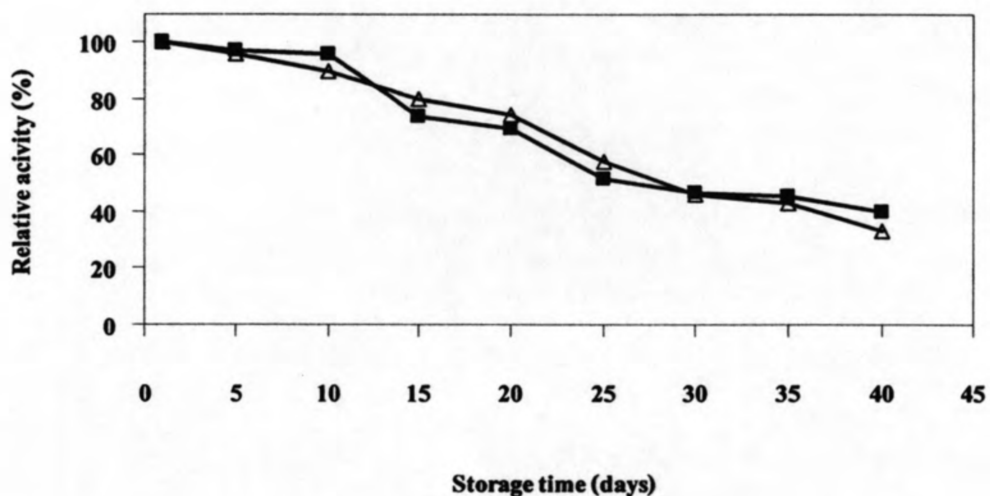


Figure 3.27 Storage stability at room temperature (A), and 4°C (B) of the free and immobilized PheDH on aminated silica. Free (■) and immobilized (△) enzyme were stored in 10 mM phosphate buffer, pH 7.4 containing 2-mercaptoethanol and glycerol as a stabilizer. Results shown were average values of duplicates experiments.

3.5.6 Batch reusability of immobilized PheDH for L-phenylalanine production

One of the most important criteria for evaluating the possibility of practical application of the immobilized enzyme is its reusability. The reusability of the immobilized PheDH was then examined by using the same systems repeatedly 3 times. As shown in **Figure 3.28**, it was found that the immobilized PheDH produced a good yield of L-phenylalanine in the successive cycles with production yield of 62.7%, 60.11% and 58.0%, respectively. The activity of first batch was taken as 100%. As can be seen in **Figure 3.28**, the silica bound PheDH retained 84% of its original activity after 3 repeated uses. **Figure 3.29** shows the result of TLC chromatogram of L-phenylalanine production from each cycle. The product yield (determined as described in section 2.14.3) was ranging between 58.0-62.7%.

3.6 Production of amino acids

In this study, immobilized PheDH was used to synthesize amino acids using their keto acids as substrates. The tested keto acids were phenylpyrubvate, α-ketocaproate, α-ketoisocaproate, α-ketovalerate and α-keto-γ-methiol-butyrate which were converted to the products including L-phenylalanine, norleucine, leucine, norvaline, and methionine, respectively. Five milligrams of immobilized PheDH were incubated with 5 μmol of each substrate in reaction mixture containing 200 μmol NH_4Cl-NH_4OH, pH 9.5, and 5 μmol NADH in the total volume of 0.5 ml for 20 hours. The products of each immobilized enzyme reactions were applied on cellulose thin-layer chromatography (TLC) by running the sample with standard amino acids in parallel. The results are shown in **Figure 3.30**, it was found that the R_f value of product from each reaction was the same with expected amino acid standard. All R_f value are shown in **table 3.3**. These results indicated that the immobilized PheDH can be used to synthesize L-phenylalanine and related amino acids from their keto acids.

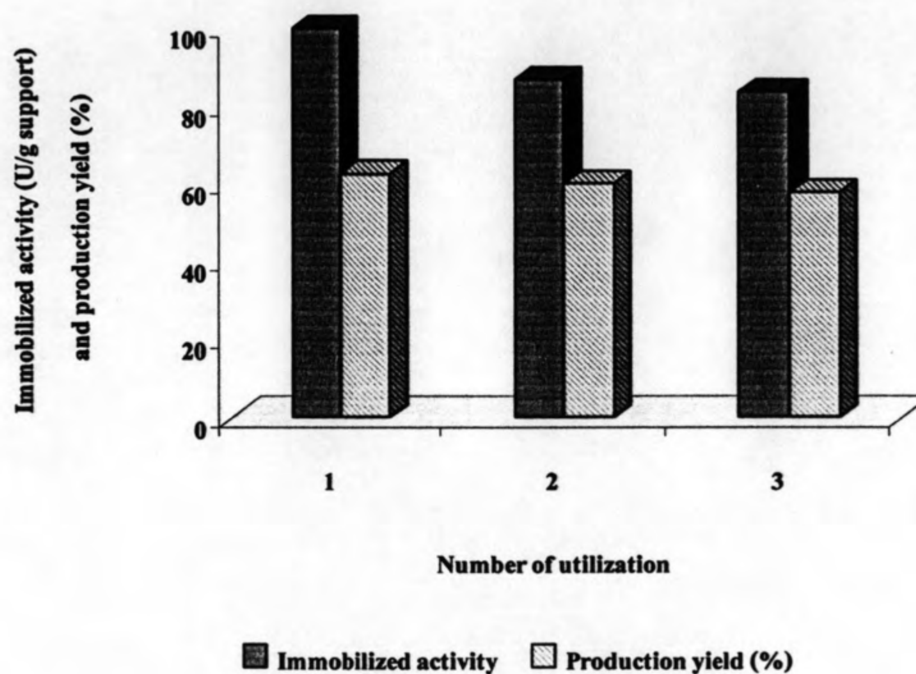


Figure 3.28 Batch reusability of immobilized phenylalanine dehydrogenase on silica for L-phenylalanine production. Reaction was performed for 6 hours at room temperature with 5 μmol phenylpyruvate, 200 μmol NH_4Cl-NH_4OH buffer (pH 8.5), 5 μmol NADH and 500 mg (wet weight) of immobilized PheDH. Immobilized enzyme was washed with 10 mM potassium phosphate buffer, pH 7.0 prior to the next production cycle.

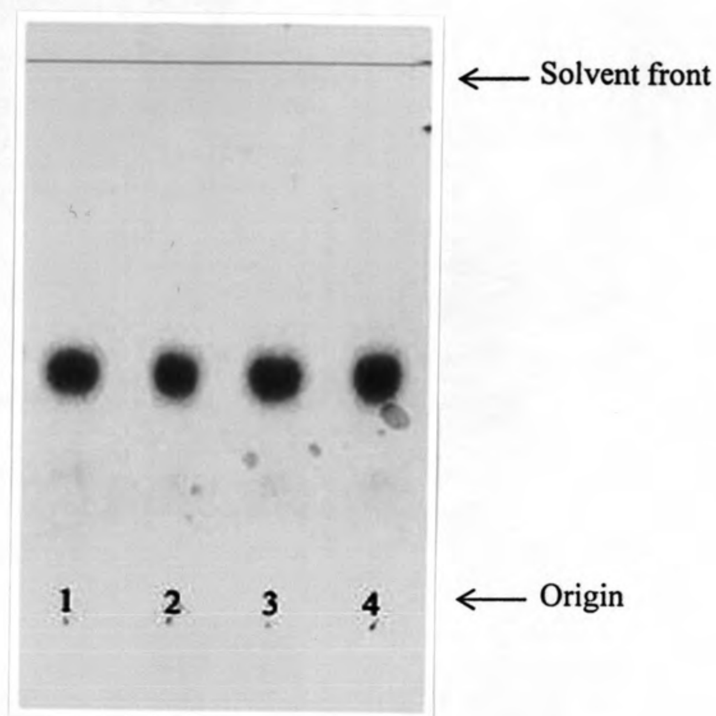


Figure 3.29 TLC chromatogram illustrating the production of L-phenylalanine.

The reaction mixture comprised of 5 μmol phenylpyruvate, 200 μmol NH_4Cl-NH_4OH buffer (pH 8.5), 5 μmol NADH, 500 mg (wet weight) of the immobilized PheDH and incubated for 6 hours.

Lane 1 = standard phenylalanine

Lane 2 = reaction mixture from the 1st cycle of L-phenylalanine production

Lane 3 = reaction mixture from the 2st cycle of L-phenylalanine production

Lane 4 = reaction mixture from the 3st cycle of L-phenylalanine production

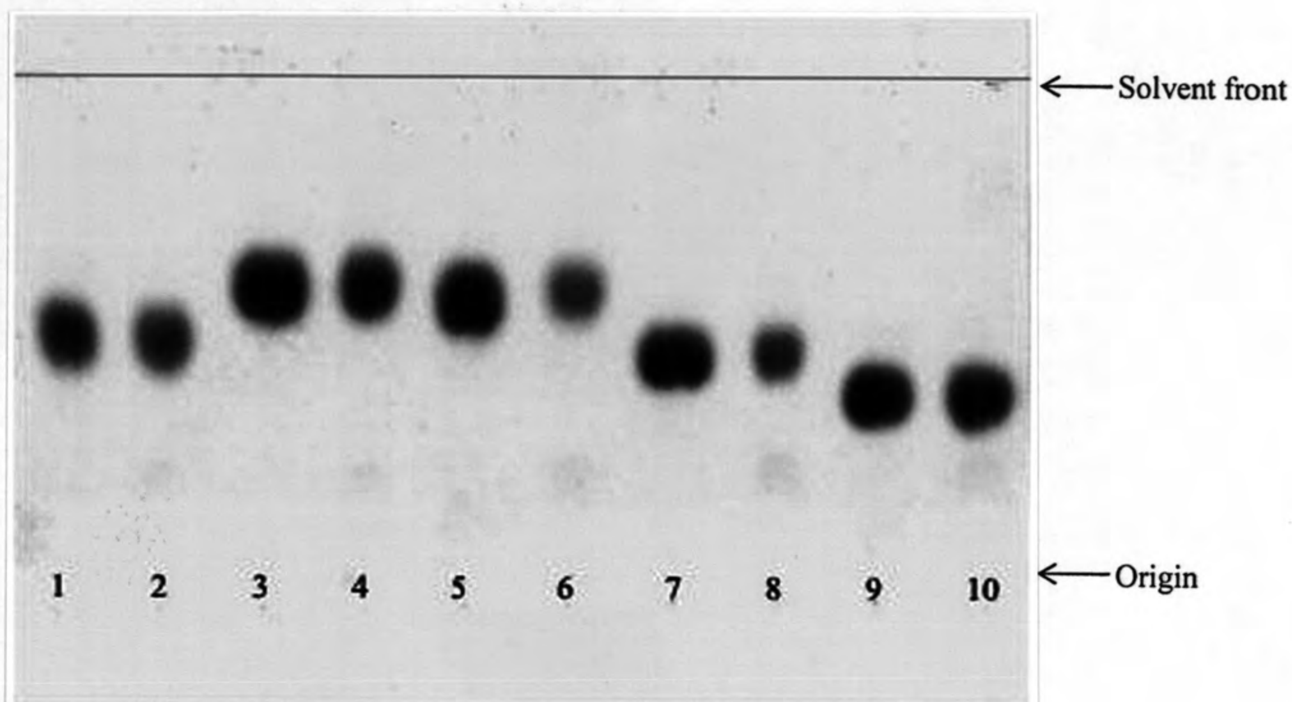


Figure 3.30 TLC analysis of the products catalyzed by the immobilized phenylalanine dehydrogenase. Five milligrams (wet weight) of immobilized PheDH on silica was incubated with the mixture containing 5 μmol of substrates, 200 μmol of NH_4Cl-NH_4OH, pH 9.5 and 5 μmol of NADH at 30°C for 20 hours. The products were applied on TLC plate. After developed with solvent, the ninhydrin solution was sprayed to invisible spot of amino acids to form purple color.

Lane 1 = standard phenylalanine
Lane 2 = phenylalanine sample
Lane 3 = standard norleucine
Lane 4 = norleucine sample
Lane 5 = standard leucine
Lane 6 = leucine sample
Lane 7 = standard norvaline
Lane 8 = norvaline sample
Lane 9 = standard methionine
Lane 10 = methionine sample

Table 3.3 R_f value of product from each reaction separated by TLC

Lane	Reaction	R_f value
1	L-phenylalanine standard	0.55
2	product from phenylapyruvate reaction	0.55
3	L-norleucine standard	0.63
4	product from α-ketocaproate reaction	0.63
5	L-leucine standard	0.62
6	product from α-ketoisocaproate reaction	0.62
7	L-norvaline standard	0.51
8	product from α-ketovalerate reaction	0.51
9	L-methionine standard	0.45
10	product from α-keto-γ-methiol-butyrate reaction	0.45

The amount of L-amino acids produced from the immobilized enzyme reactions were determined by TLC techniques. The TLC-intensity calibration curve was linearly expressed as shown in Appendix G. Each amino acid was run in parallel with 5-25 μmol of their standards as described in section 2.14.3. L-Phenyalanine content was calculated from the standard curve to be 3.5 μmol which was 70.9% conversion. Norleucine, leucine, norvaline and methionine were produced with 96.7%, 80.4%, 62.5% and 100% conversion, respectively.