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# Immobilization of Phenylalanine Dehydrogenase and Its Application in Flow-Injection Analysis System for Determination of Plasma Phenylalanine

**Leman Tarhan · Hulya Ayar-Kayali**

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**Abstract** Phenylalanine dehydrogenase (L-PheDH) from *Sporosarcina ureae* was immobilized on DEAE-cellulose, modified initially with 2-amino-4,6-dichloro-s-triazine followed by hexamethylenediamine and glutaraldehyde. The highest activity of immobilized PheDH was determined as 95.75 U/g support with 56% retained activity. The optimum pH value of immobilized L-PheDH was shifted from pH 10.4 to 11.0. The immobilized L-PheDH showed activity variations close to the maximum value in a wider temperature range of 45–55 °C, whereas it was 40 °C for the native enzyme. The pH and the thermal stability of the immobilized L-PheDH were also better than the native enzyme. At pH 10.4 and 25 °C, $K_m$ values of the native and the immobilized L-PheDH were determined as $K_{m\ Phe}$=0.118, 0.063 mM and $K_{m\ NAD^+}$=0.234, 0.128 mM, respectively. Formed NADH at the exit of packed bed reactor column was detected by the flow-injection analysis system. The conversion efficiency of the reactor was found to be 100% in the range of 5–600 μM Phe at 9 mM $NAD^+$ with a total flow rate of 0.1 mL/min. The reactor was used for the analyses of 30 samples each for 3 h per day. The half-life period of the reactor was 15 days.

**Keywords** Phenylketonuria · Phenylalanine dehydrogenase · Flow-injection system · Packed bed reactor · Immobilization

## Introduction

Phenylketonuria (PKU) is an inborn error of amino acid metabolism in which the conversion of L-phenylalanine (L-Phe) to L-tyrosine is impaired and can cause profound mental retardation if not detected and treated soon after birth [1]. This disease is due to an autosomal recessive inheritance that codes for a type of phenylalanine hydroxylase with reduced enzymatic activity, resulting in high levels of L-Phe in inborn fluid and phenylpyruvate in urine [2].

L. Tarhan (✉) · H. Ayar-Kayali
Faculty of Science and Arts, Department of Chemistry, University of Dokuz Eylül, 35160 Buca, Izmir, Turkey
e-mail: leman.tarhan@deu.edu.tr

Early quantitative measurement of the plasma L-Phe is essential for the diagnosis of PKU and the control of dietary therapy of the patients [3, 4]. In this sense, diagnosis of PKU has drawn special attention [5–7]. This disease is generally detected in neonatal screening through the bacterial inhibition assay, but this method has low analytical sensitivity and poor precision [8]. During the past decade, analytical L-Phe level assessment procedures that use fluorimetry [9], high-performance liquid chromatography [10], or tandem mass spectrometry have been developed and used for screening PKU extensively [11]. However, these methods are not suitable for routine clinical analysis, as they are time consuming and require complex, tedious sample preparation. They also need highly sophisticated instrumentation and therefore are inconvenient and economical. An alternative method is based on spectrophotometric determination of L-Phe by using $NAD^+$-dependent phenylalanine dehydrogenase (PheDH) detected in *Brevibacterium* sp. [12]. This enzyme constitutes a valuable analytical tool for the colorimetric screening of PKU [13] and has been also proposed as biocatalyst for the enantioselective synthesis of L-Phe [13, 14]. However, the application of L-PheDH is practically limited by its rapid temperature-dependent inactivation [15]. In order to increase its stability and economical application, special attention is currently devoted on the modification and the immobilization of L-PheDH. For instance, L-PheDH immobilization on tresylated poly(vinyl alcohol) beads [16], modification with cyclodextrin derivatives [17], biosensor coated amino-activated cellulose membrane by cross-linking with glutaraldehyde (GDA) [18], L-adamantanyl modified L-PheDH immobilization on β-cyclodextrins coated Au electrode [19], and carbon paste electrode with 3,4-dihydroxybenzaldehyde as an electron mediator [20] can be given as examples to the limited research in this area.

The aim of this study was the immobilization and characterization of L-PheDH from *Sporosarcina ureae* on modified DEAE-cellulose and its application in a flow-injection system contained in a packed bed reactor for determination of L-Phe level in plasma which enzymatic reaction involved via spectrophotometer.

## Materials and Methods

### Modification of DEAE-Cellulose and Immobilization of L-PheDH

DEAE-cellulose was treated initially with 2-amino-4,6-dichloro-s-triazine followed by hexamethylenediamine and GDA under optimum conditions, as described in [21].

Immobilization of L-PheDH from *S. ureae* on modified DEAE-cellulose (20 mg) as a support was carried out in a total volume of 1 mL with varying amounts of L-PheDH in 50 mM phosphate buffer at pH 7.5 by shaking at +4 °C, overnight. This procedure was repeated in the presence of 2% of GDA as a cross-linking agent.

To elute the free and adsorbed enzyme, the immobilized L-PheDH samples were washed initially with KCl of increasing and then decreasing ionic strengths, prepared in 50 mM phosphate buffer, at pH 7.5, until no absorption at 280 nm was detected in the effluent. The amount of covalent bound L-PheDH on the modified support was calculated using the deference between the protein amount at the beginning and elusion medium with Bradford method by using bovine serum albumin as standard [22].

### Activity Measurements for Native and Immobilized L-PheDH

The enzymatic activities of native L-PheDH (1.4.1.20) were measured in a 1.0-mL reaction mixture 100 mM glycine/KCI/KOH, pH 10.4, containing 2.5 mM $NAD^+$ and 10 mM L-Phe

at 340 nm and 30 °C [23]. The activity of the native L-PheDH was 9.1 U $mg^{-1}$ $min^{-1}$ under standard assay condition.

The enzymatic activities of immobilized L-PheDH (20 mg) were measured also under the same condition after incubation in 4 mL of the reaction medium for 5 min. At the end of this period, they were removed quickly by vacuum filtration.

One international unit is defined as micromole NADH formed per minute under defined assay conditions by the oxidative deamination activity of 1 mg native or 1 g immobilized PheDH.

Plasma of blood samples was taken from the PKU patients whose diets are periodically controlled at the Hospital of Dokuz Eylul University. To obtain the plasma, the blood samples in 1.8 mg $ml^{-1}$ EDTA contained tube were centrifuged at 3,000 rpm (4,000×*g*) for 10 min. In the following step, the plasma samples were deproteinized with an equal volume of perchloric acid (1.5 mol/L) and centrifuged at the same condition. The obtained supernatant was stored at −86 °C. The application of the deproteinized samples in the flow-injection analysis (FIA) system was carried out after the samples were filtered through an ultrafiltration membrane (nominal molecular weight cutoff, 10,000).

At the end of the daily studying period of the FIA system, the immobilized L-PheDH in the reactor was washed with 10% glycerol in 50 mM phosphate buffer at pH 7.5 and stored in a refrigerator until use.

The results were controlled by Biochrom 30 using an autoloader in the waste samples after applications in the FIA system.

## Flow System and Procedure

In the flow cell system, substrate or plasma samples (50 μL) were pumped by a peristaltic pump (0.1 mL $min^{-1}$) via an automatic injector. Phosphate buffer streams carrying L-Phe and $NAD^+$ were passed through a column (30×3 mm, i.d.) packed with immobilized L-PheDH at 25 °C after being mixed (Fig. 1). NADH level formed in the packed bed reactor was measured with

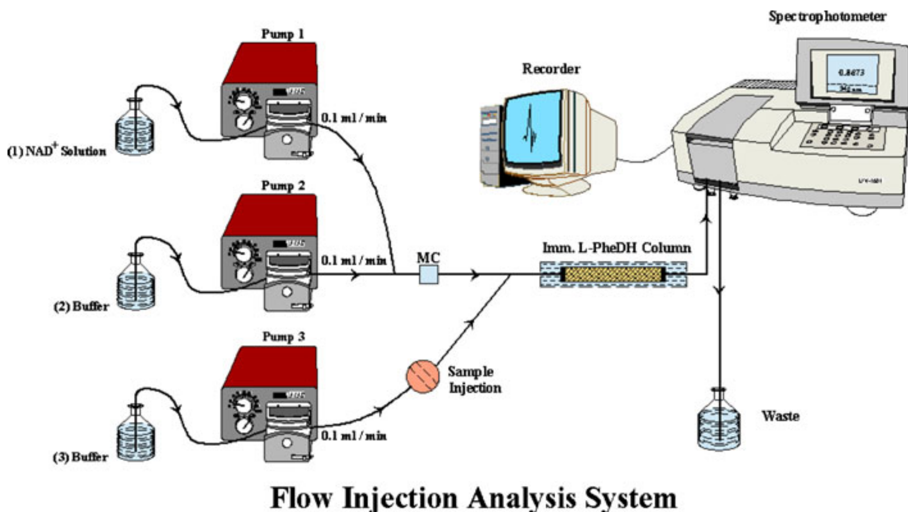


**Fig. 1** Schematic diagram of flow-injection analysis system (FIA) for determination of L-Phe. (*1*) 10 mM $NAD^+$ solution in water; (*2*) 2% (*v*/*v*) glycerol in phosphate buffer solution for dilution of $NAD^+$; (*3*) sample carrier buffer solution; *MC* mixing coil; sample injection (50 μL); immobilized L-PheDH column (30×3 mm, i.d.) in water-bath at 25 °C; spectrophotometry with a flow cell (150 μL)

spectrophotometer at 340 nm in the flow-injection system (150 μL). Formed NADH level at the exit of the packed bed reactor was measured by spectrophotometer with the flow-through cell (150 μL) at 340 nm.

### Precision, Accuracy, and Sensitivity

The precision and accuracy of this method were evaluated using quality control samples in the range of 5–600 μM phenylalanine. For intra-day assay precision and accuracy, five replicates of quality control samples at each concentration were assayed all at once within a day. The inter-day assay precision and accuracy were determined by analyzing the quality control samples on five different days. Five replicates at each concentration were assayed per day. The lower limit of quantification was determined for phenylalanine at five times.

## Results and Discussion

In the present work, immobilization and characterization of L-PheDH on modified DEAE-cellulose and then its application in FIA system were carried out in order to determinate the L-Phe level in PKU plasma.

A series of immobilizations were performed with 20 mg of modified DEAE-cellulose and varying amount of L-PheDH (0.25–1.0 mg) with and without GDA to find the optimum modified support/enzyme ratios. The results showed that the immobilized L-PheDH activity first increased up to certain values and then decreased slowly (Fig. 2). Under the standard assay condition, the highest activity of immobilized L-PheDH was 95.75 U/min $g_{support}$, with 56% retained activity and 10.52 mg/$g_{support}$ active bounded L-PheDH amount for the immobilization condition in the ratio of 20 mg support/0.6 mg enzyme without GDA. Nevertheless, these values decreased to 33% retained activity and 5.45 mg/$g_{support}$ active bounded L-PheDH amount after the immobilization with GDA. Decreases in the activity of

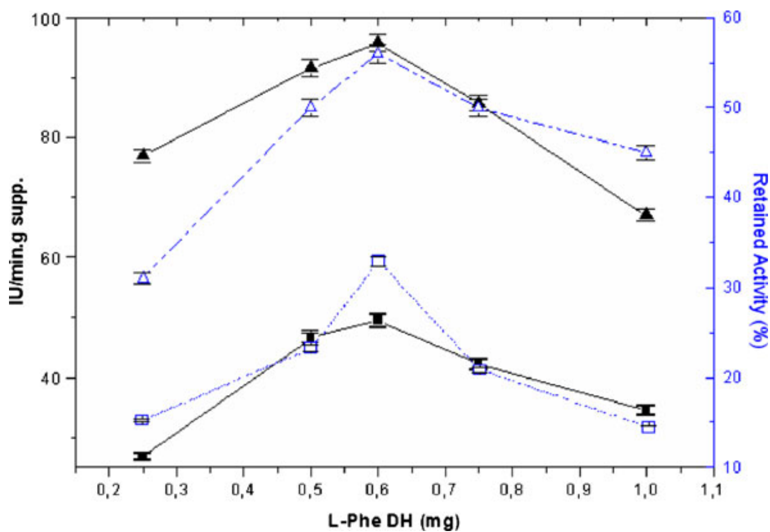


**Fig. 2** Activity and retained activity variations of immobilized L-PheDH with respect to the enzyme amount, without GDA (*closed* and *open triangles*) and in the presence of GDA (*closed* and *open squares*)

immobilized L-PheDH in spite of the increase in the amount of the enzyme can be explained by sterical interactions [12, 24]. Furthermore, the decreases in retained activity of the immobilized L-PheDH in the presence of GDA indicate that the cross-linking with GDA was affected close to the support matrix, which lead to the sterical hindrances arising from the multipoint attachments and therefore lowering of conformational stability. This situation was also supported by lower stability properties of the immobilized L-PheDH without GDA compared to the sample with GDA. The following study was continued with immobilized samples, which was prepared without GDA.

## pH-Dependent Activity and Stability Variations

The pH-dependent variation of L-PheDH activity from *S. ureae* was investigated at 30 °C for different pH values in 50 mM phosphate (pH 6.5–8.5), borate/NaOH (pH 9.5), glycine/KCl/KOH (10.4 and 10.8), and hydrogen orthophosphate/NaOH (pH 11.0, 11.3) buffers under previously defined standard assay conditions for the native and the immobilized L-PheDH.

The activity of both native and immobilized L-PheDH was increased sharply after pH 8.5. The optimum pH value of the immobilized PheDH was shifted from pH 10.4 to 11.0 that is in the more alkali region when compared to native L-PheDH of *S. ureae*. This shows that the immobilization is achieved between GDA on modified support and ε-$NH_2$ groups of lysine and N-terminal amino acid of L-PheDH, causing the total negative charge to increase. The situation leads to accumulation of more protons in the microenvironment that shifts the optimum pH to the alkaline region [25, 26]. The optimum pH level of L-PheDH was 10.5 for *S. ureae* SCRC-RO4 but dependent on the source, e.g., 12.0 for *Microbacterium* sp., 10.8 for *Rhodococcus maris*, 10.5 for *Brevibacterium* sp. SCRC-R79a, and 10.0 for *Nocardia* sp. 239 [22, 23, 27, 28].

Retained activity measurements for the native and the immobilized L-PheDH on modified DEAE-cellulose were made under standard assay conditions after incubation in 50 mM buffers of different pH values at 30 °C for 15 min. As shown in Fig. 3b, the pH stability of the immobilized L-PheDH was higher than the native at 30 °C and for 15 min incubation period. The retained activities of immobilized L-PheDH incubated at pH 8.5, 9.5, and 10.4 were 1.66-, 2.85-, and 5.45-fold higher than native enzyme, respectively. According to the results, enzymatic activity of the native and the immobilized L-PheDH incubated at pH 7.5 did not change significantly up to 5 and 56 h, respectively. All results showed that the immobilization of L-PheDH on the hydrophilically modified DEAE-cellulose leaded to greater pH stability when compared to the native enzyme.

## Temperature-Dependent Activity and Stability Variations

Temperature-dependent activity variation of the native enzyme was investigated in 50 mM phosphate buffer, at pH 7.5 for different temperatures.

Native L-PheDH from *S. ureae* showed maximum activity at 40 °C. According to some researchers, optimum temperature yielding maximum L-PheDH activity differs with respect to source, and it was 50 °C for *Bacillus badius* and 70 °C for *Microbacterium* sp. strain DM 86-1 [15, 22]. In comparison with the native enzyme, the maximum activity of immobilized L-PheDH was showed in a wider temperature range, i.e., 40–55 °C (Fig. 4a). This situation suggested that the conformation rigidity of L-PheDH polypeptide chains was increased after immobilization, therefore requiring higher temperature for expressing its maximum catalytic activity. The higher thermal stability of immobilized L-PheDH in the range of 40–55 °C, where the maximum activity was observed, indicated the increases in conformational rigidity compared to the native enzyme.

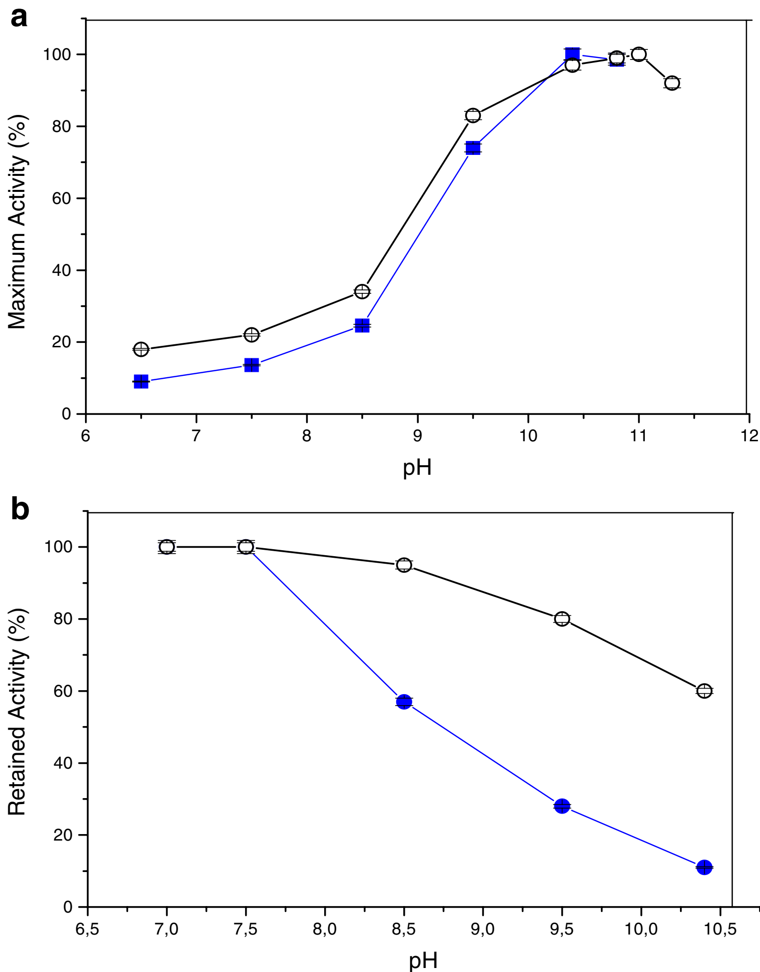


**Fig. 3** **a** pH-dependent activity variations of native (*closed circles*) and immobilized L-PheDH (*open circles*) at 30 °C. **b** pH-dependent stability variations of native (*closed circles*) and immobilized L-PheDH (*open circles*) at 30 °C for 15 min

Kinetic constants of both native and covalently bound L-PheDH catalysis have a temperature dependence of Arrhenius form. Activation energy of the native and the immobilized L-PheDH was determined at similar values as 44.48 and 48.04 kJ $mol^{-1}$, respectively. This indicates that introduction of the substrate in to the enzyme and formation of active complex did not change the favorable electron flow between the substrate and enzyme, the polarity, or ionic concentration in the vicinity of the active site significantly.

Thermal stabilities of the native and the immobilized L-PheDH were investigated by measuring the retained activity at different temperatures ranging from 20 °C to 55 °C by incubating the enzyme during 3.5 h at pH 7.5. As can be seen from Fig. 4b, the native L-PheDH did not lose any activity during 5.5 h incubation period at 30 °C. The deactivation rate of native L-PheDH increased with the increase in the temperature after 45 °C. Although the retained activity of native enzyme was 96% after 15 min of incubation at 45 °C and

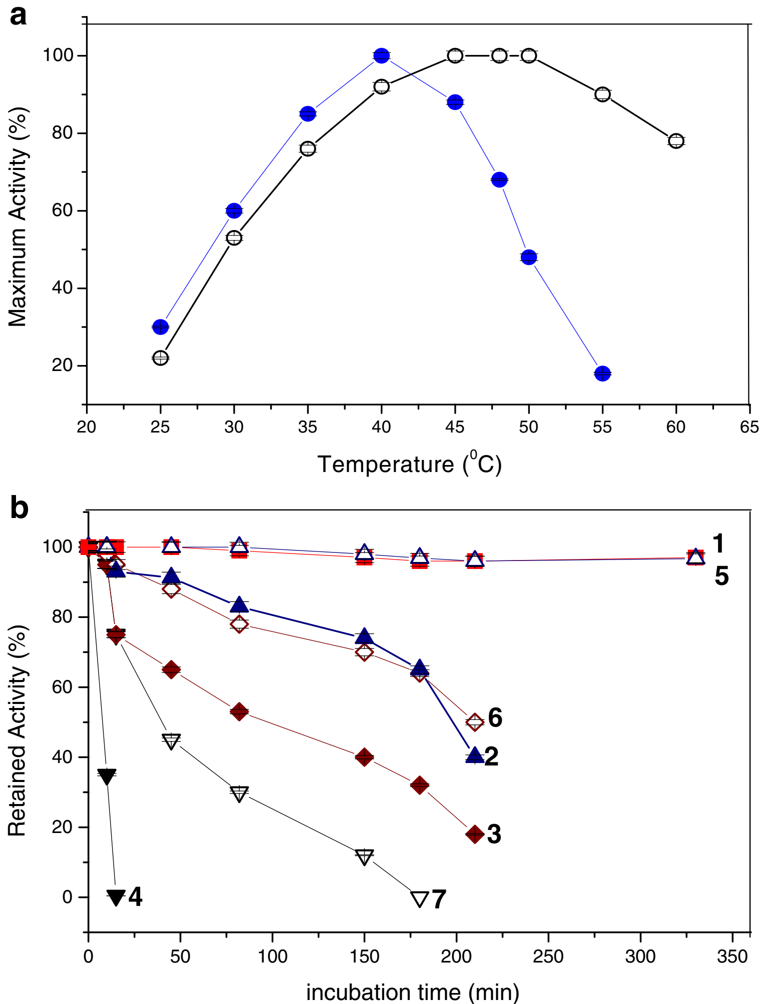


**Fig. 4** **a** Temperature-dependent activity variations of native (*closed circles*) and immobilized L-PheDH (*open circles*) at pH 7.5. **b** Temperature-dependent stability variations of native L-PheDH at pH 7.5; 30 °C (*closed circles*, *1*), 45 °C (*closed triangles*, *2*), 50 °C (*closed diamonds*, *3*), 55 °C (*closed inverted triangles*, *4*) and immobilized L-PheDH at pH 7.5; 45 °C (*open triangles*, *5*), 50 °C (*open diamonds*, *6*), 55 °C (*open inverted triangles*, *7*)

pH 7.5, it was completely inactivated at pH 10.4. A similar dependence on pH was observed at 30 °C (Fig. 3b). While the native enzyme had no loss in activity at pH 7.5, it retained only 10% of its activity at pH 10.4 (Fig. 3b). This shows that ionic and steric interactions, which are pH dependent, present major influence on the enzyme charge distribution and consequently on its stability, as compared with the temperature influence.

According to a previous research, native L-PheDH from *S. ureae* recovered 75% of its activity after incubation at pH 9.0 and 40 °C for 10 min, whereas the enzyme was completely inactivated at 50 °C [29]. However, under the same condition, the L-PheDH from *Bacillus sphaericus* showed higher thermostability and had no decrease in activity until it was heated above 55 °C [29]. Denaturation was found to occur at a higher rate with

**Table 1** Some kinetic parameters of native and immobilized L-PheDH at pH 10.4.

| Temperature | 25 °C | | | 30 °C | | |
|---|---|---|---|---|---|---|
| Kinetic parameter | $K_m$–$K_{m\ (appl)}$ (mM) | | $k_{cat}$ ($s^{-1}$) | $K_m$–$K_{m\ (appl)}$ (mM) | | $k_{cat}$ ($s^{-1}$) |
| Substrate | Phe | $NAD^+$ | Phe | Phe | $NAD^+$ | L-Phe |
| Native L-PheDH | 0.118 | 0.234 | 5.37 | 0.052 | 0.167 | 8.55 |
| Immobilized L-PheDH | 0.063 | 0.128 | 28.71 | 0.038 | 0.076 | 49.56 |

the increase in pH values of incubation medium compared to the case where only the temperature was increased. In other words, the results showed that the pH value of incubation medium is more effective than temperature in thermal denaturation rate.

L-PheDH immobilized on modified DEAE-cellulose did not lose activity up to 45 °C during all incubation periods and was also more stable than the native enzyme at the studied incubation temperatures. As shown in Fig. 4b, for incubation at 50 °C, $T_{50}$ values of the native and the immobilized L-PheDH were determined as 150 and 330 min, respectively. The results showed coherence with other research, which had shown that the immobilized L-PheDH was more resistant to heat treatment [30]. The immobilization of the enzyme with covalent bonding on hydrophilic support and the conformational stabilization due to the formation of new intra and intermolecular interactions leads to increases in the thermal stability [19, 30].

## Substrate Concentration-Dependent Activity Variations

L-PheDH activity variation was investigated with varying concentrations of L-Phe (4–13 mM) at 2.5 mM $NAD^+$ and $NAD^+$ (0.25–1.75 mM) at 10 mM L-Phe in the phosphate buffer, pH 10.4, and at 25 °C. The $K_m$ and $K_{m(appl)}$ values were determined by applying regression analysis to the Lineweaver–Burk diagrams. The decreased $K_m$, $K_{m(app)}$ and increased $k_{cat}$ values for the native and immobilized L-PheDH with the increasing temperature indicated that the reaction rates increase with the temperature due to increasing affinity of the enzyme towards both substrates (Table 1). The results showed that L-PheDH from *S. ureae* has a much lower $K_m$ for the substrates compared to the enzymes from *B. badius*, *Brevibacterium* sp., and *Rhodococcus* sp. M4 [9, 17, 27], which were 0.215, 0.4, and 0.75 mM, respectively.

The $K_{m(appl)}$ values of the immobilized L-PheDH were approximately 1.8-fold lower than that of the native L-PheDH. This indicates an increase in the affinity for Phe and $NAD^+$ and also an increase in the equilibrium constant of the Michaelis complex formation. These results show coherence with the increased affinity of L-PheDH to the substrates after immobilization as reported by researchers [31].

The retained activity, kinetic constants such $K_m$, $K_{m(app)}$ and $k_{cat}$, pH, temperature activity, and stability properties of the immobilized L-PheDH on the modified DEAE-cellulose are all quite satisfactory for its applicability especially in the reactor systems.

**Table 2** Performance parameters of the reactor.

| | CV (%) | RE (%) | Linear range (μM) at 9 mM $NAD^+$ | LLQ (μM) |
|---|---|---|---|---|
| Intra-day ($n$=5) | 2.9 to 4.5 | −1.4 to 0.84 | 5–600 | 4 |
| Inter-day ($n$=5) | 2.1 to 3.4 | −1.5 to 1.8 | | |

*CV* coefficients of variation, *RE* relative error, *LLQ* lower limit of quantification

## Reactor Performance

L-Phe levels were determined by passing the mixture of $NAD^+$ and L-Phe in 50 mM phosphate buffer at pH 7.5 through the immobilized L-PheDH column at 25 °C. The effect of $NAD^+$ concentration on the immobilized L-PheDH activity was studied over the range of 1–10 mM at the 600 μM L-Phe level and 25 °C. The conversion rate of $NAD^+$ was increased by increasing the concentration, first rapidly and then gradually. Above 7 mM, the response was almost stable. In order to prevent interference from phenylpyruvate and ammonium, 9 mM $NAD^+$ was used to provide a 3 mM concentration in the packed bed reactor. To study the specificity of this method for Phe, we assayed various amino acids (L-tyrosine, L-methionine, and L-tryptophan) at 300 μmol/L each in the reactor column system. No significant interference was detected.

The effect of flow-rate variations of the solutions was investigated in the range of 0.075–0.4 mL/min at the same value for each. The conversion efficiency of the reactor was 100% in the range of 5–600 μM Phe ($r$=0.991) at 9 mM $NAD^+$ with a total flow rate of 0.10 mL/min. The performance parameters of packed bed reactor containing immobilized L-PheDH for inter- and intra-days ($n$=5) were given in Table 2.

PKU plasma samples were diluted for quantitative determination of phenylalanine at concentrations ranging from 5 to 600 μM. In addition, measured L-Phe levels were crosschecked with the amino acid automated analyzer. The curve given by linear regression analysis was $y=0.98x+0.06$. The correlation between the L-Phe concentration and the conversion efficiency showed that the diffusion problem did not occur in this concentration range. The reactor was used for the analyses of 30 samples each for 3 h per day, then washed with 50 mM phosphate buffer containing 10% glycerol and stored at 4 °C when not in used. According to the present study, the performance of the immobilized L-PheDH on the modified DEAE-cellulose on the flow-injection cell system can be defined as an advantageous method because of the yielding accurate and precise L-Phe results.

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