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OXIDO-REDUCTIVE REACTIONS CATALYZED BY ENZYMES

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ABSTRACT

This study examines the influence of pH and temperature on the activity of three enzymes: glucose oxidase (GO), glucose 6-phosphate dehydrogenase (G6PDH), and glutamate dehydrogenase (GLuDH). The highest levels of activity for GO, GLuDH, and G6PDH were observed at 30°C, with pH values of 5.0, 7.0, and 7.5, respectively. When these enzymes were dissolved in buffers at their optimal pH levels, they remained stable at 30°C for a duration of 30 hours. Additionally, a viable NADP/NAPH interconversion process was sustained for 30 hours by employing a combination of GLuDH and G6PDH within a membrane reactor, which was intermittently supplied (at a rate of 10 mL/h) with glucose 6-phosphate (G6P) and 2- oxoglutarate/ammonium sulfate, both of which are common substrates for G6PDH and GLuDH, respectively.

INTRODUCTION

Enzymes serve as vital biological catalysts, and their functionality and stability are intricately linked to their three-dimensional macromolecular structure, characterized by four organizational levels. The primary structure encompasses the enzyme's amino acid type, sequence along the polypeptide chain, and the presence and location of disulfide bridges (S-S) if present. The secondary structure involves the interaction, via hydrogen bonds, of amino acids within the same chain or between chains when the protein comprises multiple polypeptides. The tertiary structure entails the compaction of the polypeptide chain into a three-dimensional globular form, stabilized by various weak chemical bonds, such as electrostatic interactions, dipole-dipole forces, Van der Waals interactions, hydrogen bonds, hydrophobic forces, and disulfide bridges, among others, occurring among side-chain groups of amino acid constituents. The quaternary structure arises when a protein consists of two or more interacting polypeptide chains, often referred to as subunits, each possessing distinctive

tertiary structures, and these chains are held together by weak chemical bonds. Beyond these four structural levels, there exist super-secondary structures, which are assemblies of secondary structures, and domains, which represent compactly folded amino acid regions, typically comprising 100 to 400 amino acids, found at specific loci within the protein molecule. Notably, an example of a catalytic domain is the active site of an enzyme.[1]

Enzyme catalytic activity and stability are influenced by various factors, acting on either specific sites (such as surfactants, inhibitors, and activators) or non-specific factors (such as pH, ionic strength, and temperature) within the macromolecule.[2]

pH plays a significant role in altering the ionization state of polar or ionic chemical groups present in the side chains of amino acids. It can strongly affect the non-covalent bonds responsible for stabilizing the tertiary and/or quaternary structures of proteins. When pH allows the preservation of the most suitable molecular structure for a protein, it is referred to as the optimum pH (pH_{opt}). For instance, in the case of a catalytic protein like an enzyme, maximum activity is achieved at pH_{opt}. Any deviation from this pH value may result in either a reversible (involving the partial disruption of non-essential weak chemical bonds, which can be easily reformed when pH returns to pH_{opt}) or irreversible disruption (involving extensive breakage of weak chemical bonds).[3]

Temperature serves as the energetic parameter for any system. Energy associated with temperature is distributed among molecules as kinetic energy, which increases with rising temperature. In chemical reactions, elevated temperatures lead to increased reaction rates due to more frequent intermolecular collisions. A similar principle applies to enzyme catalysis, except that beyond a certain temperature threshold, the rate of molecular denaturation − caused by irreversible rupture of non-covalent bonds, resulting in the destabilization of the overall protein structure − outpaces the reaction rate. The denaturation process occurs gradually as temperature rises, so it invariably occurs when reaction rates increase with rising temperature. However, the stabilizing effect of non-covalent bonds on the enzyme molecule diminishes with prolonged exposure to elevated temperatures, suggesting the simultaneous occurrence of activation and denaturation processes up to the temperature limit. Beyond this point, denaturation predominates over activation.[3]

In this study, we focus on three oxidative-reductive enzymes: glucose oxidase (GO; EC.1.1.3.4), glucose 6-phosphate dehydrogenase (G6PDH; EC.1.1.1.49), and glutamate

dehydrogenase (GLuDH; EC.1.4.1.3), chosen to represent this class of enzymes. The general reactions catalyzed by these enzymes can be expressed as follows:

β-D-Glucose + O_2 + **GO** → Gluconic acid + H_2O_2 + **GO**

D-glucose 6-P + NADP + **G6PDH** ⇌ 6-P-gluconolactone + NADPH + H^+ + **G6PDH**

L-Glutamate + H_2O + NAD(P) + **GLuDH** ⇌ [NAD(P)H] + **GLuDH** + α-ketoglutarate

This study aims to assess the impact of pH and temperature on the activities of GO, G6PDH, and GLuDH, as well as to investigate NADP/NADPH regeneration through the utilization of a membrane reactor, involving the combined action of G6PDH and GLuDH.

MATERIAL

The enzymes GO, G6PDH, and GLuDH, as well as coenzymes (NAD, NADP, NADH, and NADPH), were purchased from SIGMA(R). All other reagents used in the study were purchased from conventional suppliers.

METHODS

FACTORS AFFECTING ENZYME ACTIVITY AND STABILITY

Glucose oxidase (GO)

Effect of pH on activity: In a 250-mL beaker, 125 mL of 0.01 M acetate buffer with pH values of 4.0, 4.5, 5.0, 5.5, 6.0, or 6.5 were combined. The solution was continuously aerated with air and maintained at 37°C for 20 minutes in a water bath. Subsequently, 0.15 g of glucose was added with agitation at 100 rpm for 10 minutes, followed by the addition of 25 mL of an aqueous GO solution (180 U). Aliquots of 1.0 mL were extracted every 10 minutes over a 60-minute reaction period. The reaction was stopped by immersing the aliquot in a boiling water bath, and the H_2O_2 generated was measured using a spectrometer at 240 nm. The blank corresponded to a 1 mL aliquot taken prior to the addition of GO. The dissolved oxygen level (between 4 and 5 mg/mL) was measured using an oximeter (DIGIMED, DM-4).

Effect of temperature on activity: The above test was repeated at 30°C, 35°C, 40°C, and 45°C, with a pH of 5.0.

Stability at pH 5.0 and 37 °C: Buffered GO solutions (180 U) in 0.01 M acetate buffer (pH 5.0) were incubated in a water bath at 37°C for durations of 0.5 h, 1.0 h, 2.0 h, 10 h, 20 h, and 30 h. The remaining GO activity at pH 5.0 was measured as described above.

Glucose 6-phosphate dehydrogenase (G6PDH)

Effect of pH on activity: In a 1,000-µL cuvette, 910 µL of 0.05 M TRIS buffer (supplemented with 5 mM $MgCl_2$), 10 µL of G6PDH (0.1 U), 40 µL of 0.3 mM glucose 6-phosphate, and 40 µL of NADP or NAD (100 µg) were combined. The reaction was monitored using a spectrometer (λ = 340 nm) at 30°C for 60 s. Every 5 s, the amount of NADPH or NADH formed was recorded. pH values of 7.0, 7.5, 8.0, and 8.5 were investigated.

Effect of temperature on activity: The test was repeated at 30°C, 35°C, 40°C, and 45°C, with a pH of 7.5.

Effect of pH and temperature on activity: G6PDH activity was measured at temperatures of 30°C, 35°C, 40°C, and 45°C, with the pH of the reaction medium varying between 7.0 and 8.5. The composition of the medium was described earlier.

Stability at pH 7.5 and temperatures of 30 ^{o}C and 45 ^{o}C: 0.05 M TRIS (pH 7.5) buffered solutions of G6PDH (0.1 U) were incubated in a water bath at 30°C or 45°C for 30 hours. Aliquots (10 µL) were extracted every 2 hours, and the remaining activity was determined as previously described.

Glutamate dehydrogenase (GLuDH)

Effect of pH on activity: In a 1,000-µL cuvette, 720 µL of 0.05 M TRIS buffer (supplemented with 5 mM $MgCl_2$), 70 µL of 2-oxoglutarate (0.2 M), 100 µL of 3.2 M ammonium sulfate, 10 µL of NADPH or NADH (10 mg/mL), and 100 µL of GLuDH (4U) were combined. The reaction was monitored using a spectrometer (λ = 340 nm) at 30°C for 180 s. Every 30 s, the consumption of NADPH or NADH was recorded. pH values of 7.0, 7.5, 8.0, and 8.5 were examined.

Effect of temperature on activity: The test was repeated at 30°C, 35°C, 40°C, and 45°C, with a pH of 7.5.

Effect of pH and temperature on activity: GLuDH activity was evaluated at temperatures of 30°C, 35°C, 40°C, or 45°C. The pH of the reaction medium was varied between 7.0 and 8.5 for each temperature. The medium composition was as described earlier.

Stability against pH and temperature: 0.05 M TRIS (pH 7.0 or 7.5) buffered solutions of GLuDH (4U) were incubated in a water bath at 30°C or 45°C for 30 hours. Aliquots (10 µL)

were extracted every 2 hours, and the remaining activity was determined as previously described.

ANALYTICAL METHODS

Measurement of reducing sugars

The measurement of glucose 6-phosphate (G6P) was conducted using the modified Somogyi-Nelson method.[5]

Measurement of ammonia

Ammonia levels were determined using the Nessler reagent, as previously described.[6]

Measurement of hydrogen peroxide

Hydrogen peroxide was measured as previously reported.[4]

Detection of NADP(H)

The detection of potential NADP(H) release from the membrane reactor was performed on 1-mL effluent samples by measuring absorbance at 260 nm and 340 nm using a spectrophotometer.

NADP/NADPH CONVERSION IN MEMBRANE REACTOR

In a 10-mL membrane reactor (membrane cut-off of 500 Da), as described elsewhere[4], 1,000 µL of GLuDH (4 U), 100 µL of G6PDH (0.1 U), and 400 µL of NADP (10 mg/mL) were introduced. The reactor was fed for 1 hour at 10 mL/h with a 50 mM solution of G6P, followed by the addition of a solution containing 2-oxoglutarate (0.2 M) and ammonium sulfate (3.2 M), at the same feeding rate, for 2 hours. Subsequently, the reactor was alternately fed with both solutions at 2-hour intervals for a total process duration of 30 hours. Samples were collected hourly to measure ammonia and G6P consumption. The reaction was conducted at 30°C, pH 7.5, and 100 rpm. No coenzyme release from the reactor was observed.

RESULTS AND DISCUSSION

The highest activity of GO was observed at pH 5.0, with a rate of $11.5x10^{-4}$ mg H_2O_2/min.mL (Figure 1). This pH value represents the optimum condition where the enzyme attains its most favorable conformational structure, resulting from appropriate interactions of weak forces arising from the correct ionization or positioning of inducible chemical groups found

in the side chains of amino acids constituting the enzyme. This finding is consistent with existing literature.[2][7][8][9]

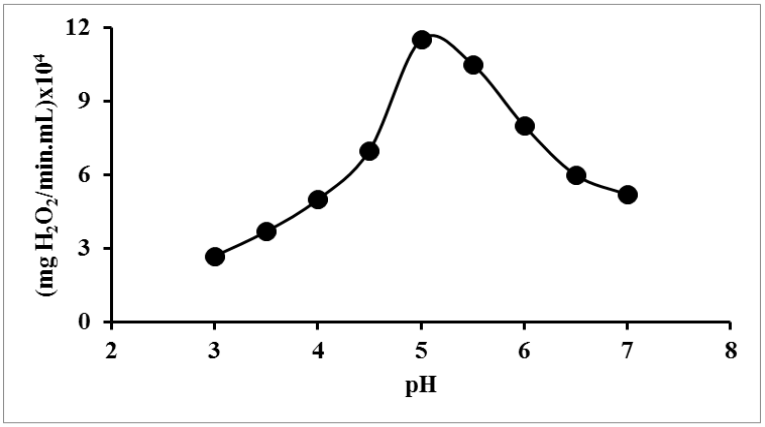

Figure 1: Effect of pH on glucose oxidase activity.

The effect of temperature on GO activity displayed an atypical profile (Figure 2). Unlike the typical bell-shaped activity/temperature profile observed for many enzymes, GO's reaction rate exhibited a linear increase with temperature between 30°C and 45°C. Furthermore, the reaction rate adhered to Van t' Hoff's law, with a doubling of the reaction rate for every 10°C increase in temperature (Figure 2).

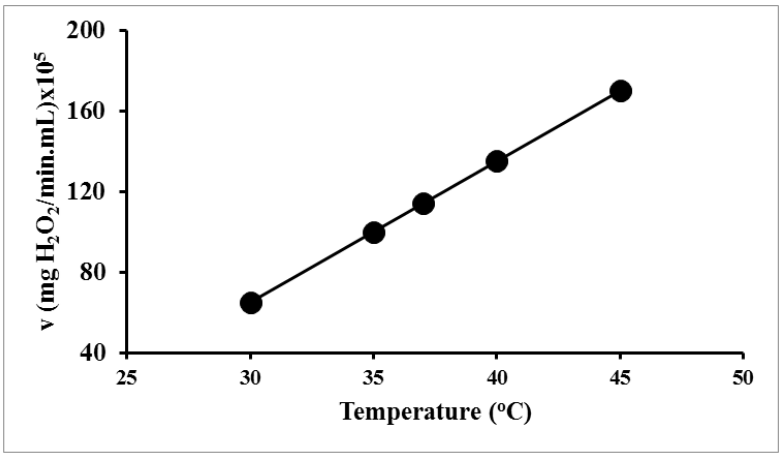

Figure 2: Effect of temperature on glucose oxidase activity. The minimal square linear equation is: $v_{GO} = 7x10^{-5}.T_{oC} – 145x10^{-5}$, (r = 0.9994).

By applying Arrhenius' method (Figure 3), the following equation was derived:

$Ln(v_{GO}) = -6{,}315.1 \times (1/T) + 13.422$ (r = 0.990) (Eq. 1)

Where v_{GO} represents the reaction rate and T denotes absolute temperature in Kelvin (K).

Considering the theoretical equations of Arrhenius' method:

$Ln(v_{GO}) = (E_a/R) \times (1/T)$ (Eq. 2)

Where E_a = activation energy (kJ/mol); and R = Clapeyron constant ($8.3144x10^{-3}$ kJ/mol.K).

$(\Delta H)_{37}{}^{o}{}_{C} = E_a - R.T$ (Eq. 3)

The comparison of equations 1 and 2 yielded E_a = 53 kJ/mol. Applying this value to equation 3 results $(\Delta H)_{37}{}^{o}{}_{C}$ = 50.4 kJ/mol.

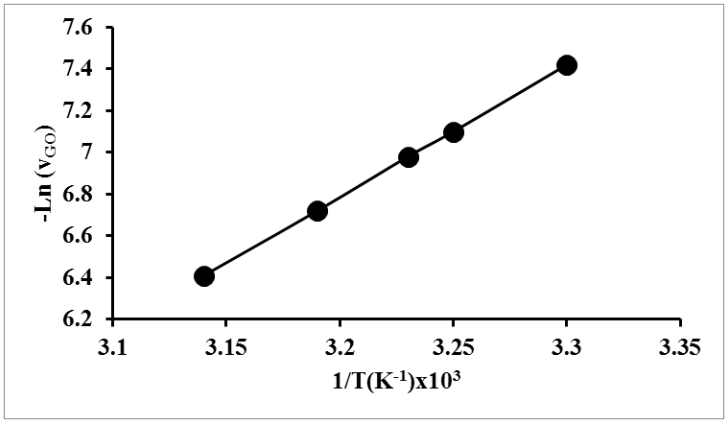

Figure 3: Calculation of GO activation energy using the Arrhenius' method.

When enzyme reactions are conducted in continuously operated reactors over extended periods, it is imperative to ascertain the stability of enzyme activity under operational conditions, primarily pH and temperature (Table 1).

Table 1: Residual glucose oxidase activity for 30 h at pH 5.0 and 37 °C.

Time (h)	v_{GO} (mg H_2O_2/min.mL)x10^3
0	1.15
0.5	1.16
1	1.17
2	1.14
10	1.01
20	0.990
30	0.985

Table 1 shows that GO activity decreased by 14% after 30 h at pH 5.0 and 37°C. The general loss of enzyme stability can be attributed to the random elimination and/or modification of non-covalent bonds within the enzyme's structure. This intensity increases over time, resulting in an overall decline in enzyme activity. Additionally, in the case of GO, the irreversible oxidation of the iron ion (N_{ox} varying from +2 to +3) may contribute to the decrease in activity.[10]

The catalysis of glucose 6-phosphate dehydrogenase (G6PDH) with G6P is dependent on the presence of either NADP or NAD coenzyme. The optimum pH (pH_{opt}) is 7.5, irrespective of the coenzyme used. However, the highest activity ($368x10^{-4}$ µM reduced coenzyme formed/s) occurs when NADP is employed (Figure 4).

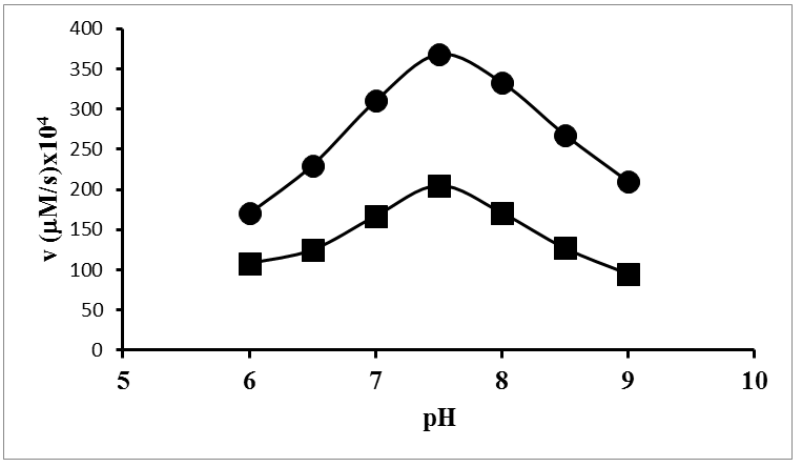

Figure 4: Effect of pH on glucose 6-phosphate dehydrogenase activity in the presence of NADP (●) and NAD (■).

A similar pattern is observed with temperature variation, although the peak activity occurs at 30°C and 35°C for NADP and NAD, respectively (Figure 5).

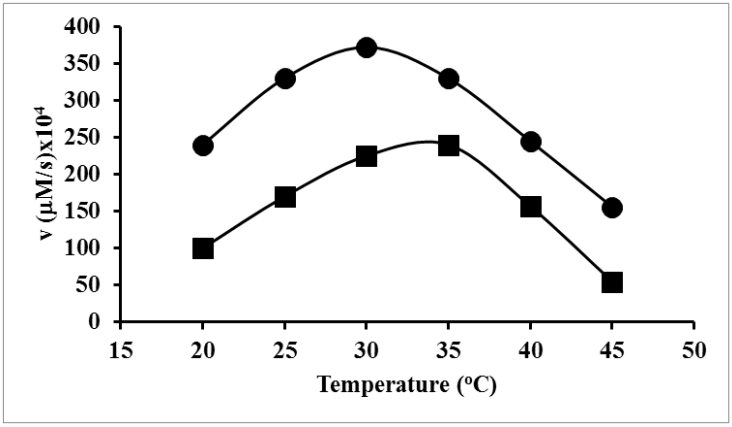

Figure 5: Effect of temperature on glucose 6-phosphate dehydrogenase activity in the presence of NADP (●) and NAD (■).

Table 2 shows the influence of pH (ranging from 7.0 to 8.5) and temperature (ranging from 30°C to 45°C) on G6PDH activity when using NADP or NAD as the coenzyme. The coefficient of variation (CV) reveals that pH has a more pronounced impact on G6PDH activity compared to temperature, independently of the coenzyme. pH affects the

G6PDH/NADP interaction profile through the ionization degree of both the enzyme and coenzyme, with the most favorable interaction occurring at pH 7.5. In contrast, temperature, which is related to the kinetic energy of molecules within the reaction system, influences the frequency of encounters between enzyme and coenzyme molecules.

Table 2: Activity of G6PDH in the presence of coenzyme (NADP* or NAD) as affected by variations in pH and temperature.**

Activity (µM/s) x10^4						
pH	**30 °C**	**35 °C**	**40 °C**	**45 °C**	**Mean**	**CV (%)**
7.0	203*/76**	186/64	182/72	169/70	185/71	8/7
7.5	278/220	253/177	265/200	338/179	284/194	13/10
8.0	236/188	276/187	256/184	221/175	247/184	10/3
8.5	199/151	187/155	199/120	192/131	194/139	3/12
Mean	229/159	226/146	226/144	230/139	-	-
CV(%)	16/39	20/39	18/41	18/37	-	-

Figure 6 shows the stability of G6PDH at pH 7.5 and temperatures of 30°C and 45°C. The enzyme exhibited instability at 45°C, losing more than 90% of its activity within 2 hours of incubation. Such behavior is commonly observed in enzymes with quaternary structures, such as G6PDH.

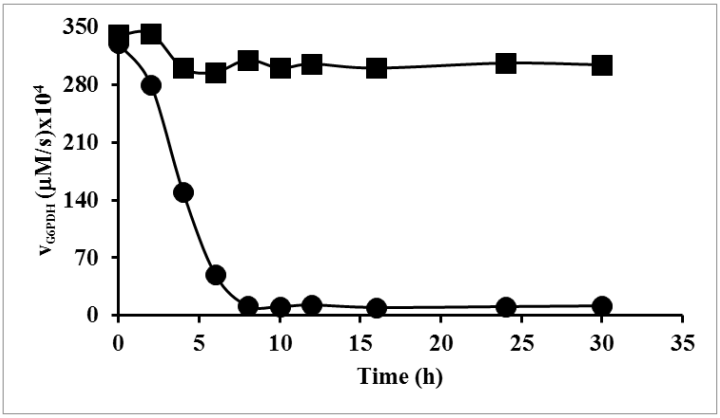

Figure 6: Stability of glucose 6-phosphate dehydrogenase at pH 7.5 and temperature [30 °C (■) and 45 °C (●)].

Utilizing Owusu and Markzoum's method, composed of equations 4-7[11], the thermal denaturation energy (E_a*), denaturation enthalpy (ΔH*), and half-life of G6PDH at 40°C and 45°C were calculated.

$\text{Log } v = - k_d.t + b$ (Eq. 4)

$\text{Log } [(k_d)_{45}/(k_d)_{40}] = (E_a*/2.303.R).[(T_{45} – T_{40})/T_{40}.T_{45})]$ (Eq. 5)

$\Delta H^* = E_a^* - R.T$ (Eq. 6)

$t_{1/2} = (Ln\ 2)/k_d$ (Eq. 7)

Where: Log v = logarithm of enzyme activity; k_d = thermal denaturation constant (h^{-1}); t = time (h); b = linear coefficient; R = Clapeyron constant ($8.3144x10^{-3}$ kJ/mol.K); T = absolute temperature (K); $t_{1/2}$ = half-life (h).

Table 3: G6PDH activity (µM/s) measured between 0 and 1.5 h in the presence of NADP at pH 7.5 and temperatures of 40 °C and 45 °C. The logarithm of activity is in parenthesis.

t	Temperature (°C)	
(h)	40	45
0	-	-
0.25	0.0342 (-1.466)	0.0240 (-1.620)
0.50	0.0326 (-1.487)	0.0160 (-1.796)
0.75	0.0318 (-1.498)	0.0112 (-1.951)
1.00	0.0309 (-1.510)	0.00600 (-2.222)
1.25	0.0298 (-1.526)	0.00390 (-2.408)
1.50	0.0288 (-1.541)	-

By applying Eq. 4 to the data of Table 3 for both temperatures:

$Log\ v_{40} = - 0.059.t - 1.45$ (Eq. 8)

$Log\ v_{45} = - 0.80.t - 1.41$ (Eq. 9)

With $(k_d)_{40} = 0.059\ h^{-1}$ and $(k_d)_{45} = 0.80\ h^{-1}$, equations 5-7 yield $E_a^* = 423$ kJ/mol, $\Delta H^* = 429$ kJ/mol, $t_{1/2(40)} = 11.7$ h and $t_{1/2(45)} = 0.90$ h.

A ΔH^* in the range of 200 to 300 kJ/mol indicates the unfolding of tertiary and/or quaternary protein structures.[11] Clearly, at 45°C, the quaternary structure of G6PDH is irreversibly unfolded. Considering the obtained half-lives for G6PDH, it is advisable to maintain the operational temperature below 40°C.

The profiles of activity-pH curves for GLuDH are atypical independently of the coenzyme (NADPH or NADH) (Figure 7). The oscillations at pH 7.0 to 8.5 were already described in the literature.[12] The highest GLuDH activity occurred at pH 7.0 in presence of NADPH (0.35 mg NH_4^+ consumed/mL.s) or NADH (0.50 mg NH_4^+ consumed/mL.s). Probably, this behavior is related to the complex quaternary structure of GLuDH – the enzyme has six polypeptide chains that must fit each other in specific spatial positions to accommodate the coenzyme and the substrates (2-oxoglutarate and ammonium ion)[13] –, which is sensitive to hydrogen ion concentration. The formation and/or disruption pattern of non-covalent bonds

that stabilizes the GLuDH molecule is reversibly affected by pH variation between 7.0 and 8.5.

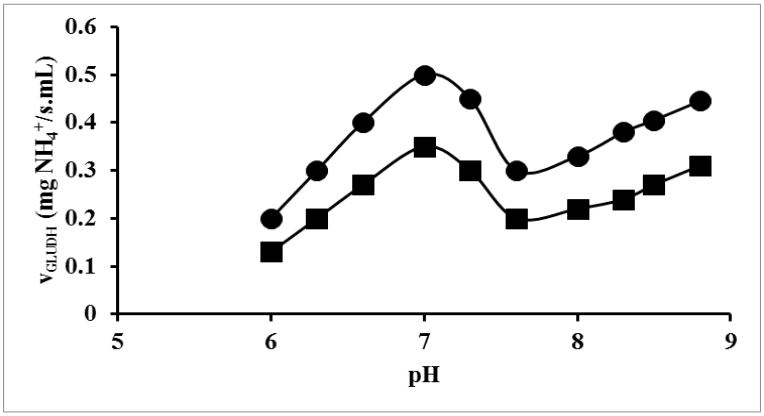

Figure 7: Effect of pH on glutamate dehydrogenase activity in the presence of NADPH (■) and NADH (●).

GLuDH activity increased as temperature ranged from 30°C to 45°C. Furthermore, the activity adhered to van t' Hoff's law (Table 4). By applying Arrhenius' method, an activation energy (E_a) of 53 kJ/mol was determined for GLuDH in the presence of NADPH.

Table 4: Effect of temperature on the activity of glutamate dehydrogenase.

Temperature (^oC)	Activity (mg NH_4^+/mL.s)
30	0.0264
35	0.0424
40	0.0584
45	0.0744

Table 5 shows the combined effect of pH and temperature on GLuDH activity. Similar to G6PDH, pH exerted a more significant influence on activity than temperature, independently of the coenzyme used. The explanation offered for G6PDH can be extended to GLuDH.

Table 5: Simultaneous effect of pH and temperature on GLuDH activity in the presence of coenzymes (NADPH* or NADH).**

Activity (mg NH_4^+consumed/mL.s)x10^3						
pH	**30 ^oC**	**35 ^oC**	**40 ^oC**	**45 ^oC**	**Average**	**CV (%)**
7.0	358*/163**	321/446	330/462	320/397	332/367	5.3/38
7.5	42/35	43/38	53/62	69/72	52/52	24/35
8.0	199/286	173/215	212/178	320/309	226/247	29/25

8.5	141/330	126/287	94/276	139/142	125/259	17/31
Average	185/207	166/247	172/245	212/230	-	-
CV (%)	72/65	70/69	73/69	60/65	-	-

Figure 8 illustrates the stability of GLuDH at pH 7.0 for 30 h at temperatures of 30°C and 45°C. The enzyme remained stable under these conditions.

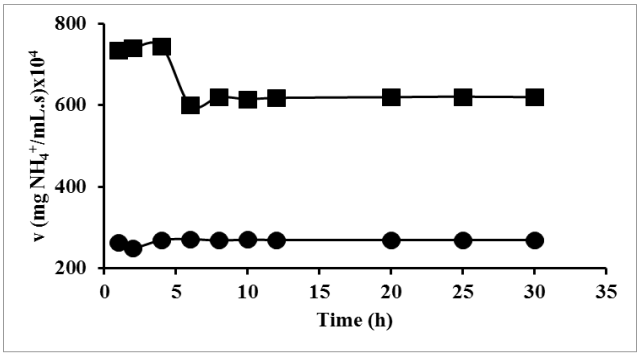

Figure 8: Stability of GLuDH for 30 h at pH 7.0 and 30 °C (●) and 45 °C (■).

In industrial applications, using coenzyme-dependent oxidative enzymes is challenging due to their high cost, necessitating coenzyme regeneration during the process. Based on a previous study, coenzyme regeneration using the G6PDH/GLuDH system yielded promising results.[6] Therefore, an experiment was devised to continuously regenerate NADP/NADPH by feeding a membrane reactor containing both enzymes and NADP with alternating additions of G6P and 2-oxoglutarate plus ammonium sulfate. The results are depicted in Figure 9. The concentrations of substrates (G6P and ammonium ion) oscillated during the process, indicating that the oxidized (NADP) and reduced (NADPH) forms also fluctuated. Due to the transient nature of the continuous process, it was challenging to calculate an overall mean conversion for each substrate (G6P and ammonium ion). Nonetheless, an estimate was attempted by considering 10-hour intervals (Table 6). The percentage of substrate conversions was less than 50%, suggesting that the process requires further refinement, even though NADP/NADPH interconversion did occur to some extent.

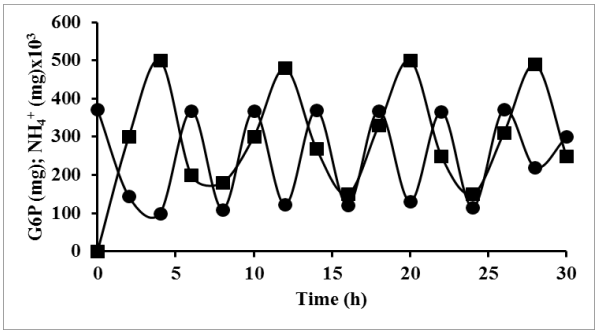

Figure 9: Variation of glucose 6-phosphate (●) and ammonia (■) over 30 h due to the combined action of G6PDH and GLuDH in the presence of NADP. The reaction conditions included feeding rate of 10 mL/h, pH 7.0, and 30 °C.

Table 6: Intervals indicating the percentage of mean conversion of glucose 6-phosphate and ammonia during the continuous process catalyzed by the G6PDH/GLuDH system.

Time interval (h)	G6P (%)	Ammonium (%)
1 – 12	47	48
13 – 24	20	14
25 – 30	-	27

CONCLUSION

The activities of GO, G6PDH, and GLuDH are significantly influenced by pH and temperature. Their optimal conditions are pH 5.0/37°C, pH 7.5/30°C, and pH 7.0/45°C, respectively. When considering the combined effects of pH and temperature on enzyme activities, pH exerts a more pronounced influence than temperature. Furthermore, employing the G6PDH/GLuDH system in a continuously operated membrane reactor demonstrates the feasibility of interconverting NADP and NADPH for a minimum of 30 hours.

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