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B-NICOTINAMIDE ADENINE COENZYMES IMMOBILIZED ON ANIONIC RESIN: EVALUATION OF DETACHMENT BY SPECTROPHOTOMETRY AND CONDUCTIMETRY

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ABSTRACT

The coenzymes NAD, NADH, NADP, and NADPH were immobilized on anionic resins. The most suitable resin/coenzyme pairs were selected by varying the type of resin (in terms of percentage of cross-linking and bead size), amount of resin, and the pH of the immobilization medium. The stability of the resin/coenzyme complex was evaluated by measuring the conductivity of the aqueous suspension after stirring for 30 h at pH 6.0 or 9.0.

KEYWORDS: Immobilization, Coenzymes, Anion exchange resins, Conductivity.

INTRODUCTION

Coenzymes such as β-nicotinamide adenine derivatives – NAD(H) and NADP(H), for example – are soluble compounds that link to enzymes in the occurrence of catalysis. Approximately one fifth of known enzymes are coenzyme-dependent, with dehydrogenases and several oxidoreductases among them. [1]

As coenzymes suffer irreversible oxidation or reduction during catalysis and present a high cost, the industrial use of coenzyme-dependent enzymes in the production of fine chemicals (for instance, chiral compounds) is hindered.[2] Both problems can be circumvented by using coupled reaction (based on the principle of redox reversion, using a cheaper co-substrate) and by immobilizing the coenzyme on inert support (its reuse after finishing the reaction leads to a cost reduction). The immobilization follows the same reasoning applied to the immobilization of enzymes, cells, or organelles.[3] Among the types of immobilizations, the

most adequate are those involving binding on supports (adsorption, covalent bonding, and cross-linking).

In this study, β-nicotinamide adenine coenzymes were adsorbed on anionic resins (polystyrene- divinylbenzene copolymers). The resins were chosen considering the practicality of the adsorption method, the ease of handling the coenzyme-resin complex, the plentiful availability in the market, the non-toxicity, and the low cost. The reuse of the resins when desired and the separation of the coenzyme from the support are performed simply by changing the medium ionic strength.[4,5]

MATERIAL AND METHODS

The cofactors [NAD (P) and NAD (P) H] and the anion exchange resins were purchased from Sigma® (St. Louis, MO, USA). All the other reagents (P.A. grade) were purchased from traditional suppliers.

Immobilization of coenzymes

Resins with different cross-linking percentages (1x2, 1x4, and 1x8) and beads sizes (100, 200, and 400 mesh) were weighed (25, 50 or 100 mg). The beads were rinsed with 25 mL deionized water with pH adjusted to 7.5 or 8.5. The suspension was maintained in a shaker at 30°C and 100 rpm for 24h, following centrifugation (3000*g*/10 min). The supernatant was discharged, and the beads were resuspended with 20 mL deionized water (pH adjusted between 7 and 8.5), followed by the addition of a volume of an aqueous solution of coenzyme ($2.22x10^{-5}$ M, with pH adjusted between 7 and 8.5). The new suspension was maintained under stirring of 100 rpm at 30°C for 4h. The percentage of immobilization efficiency (IE) was calculated by the following equation:

$IE = [(A_o – A_1) \div A_o].100$ (Eq. 1)

Where: A_o = initial absorbance of the coenzyme solution; A_1 = absorbance of the supernatant after immobilization.

The absorbances were read using a Beckman-Coulter spectrophotometer, with the molecular extinction coefficient (ε) for each coenzyme being, namely: $(NAD/NADP)_{260nm}$ = 18,000 $L.mol^{-1}.cm^{-1}$, $(NADH/NADPH)_{260nm}$ = 15,000 $L.mol^{-1}.cm^{-1}$, and $(NADH/NADPH)_{340nm}$ = 6,220 $L.mol^{-1}.cm^{-1}$.

Determination of coenzyme desorption

The coenzyme/resin complex was suspended in 25 mL distilled water (pH adjusted to 6.0 or 9.0), followed by stirring (300 rpm) at 37 ^{o}C for 72 h. The suspension was centrifuged (3,000*g*/10 min) and a sample of the supernatant was collected for analytical purposes.

Conductivity of coenzyme solutions

In a conductivity cell (TECNAL®; mod: TEC-MP4; São Paulo, Brazil), 40 mL of buffer solution (1 mM phosphate buffer; pH 6.0, 7.5, or 9.0) or coenzyme solution, at concentrations of 0.5, 1, 2, 3, 4, and 5 mM, were poured and the conductivity was measured ($\mu S.cm^{-1}$). A standard curve relating the coenzyme concentration versus conductivity was obtained.

The immobilization efficiency may also be calculated by conductimetry through the following equation:

$IE = [(C_o - C_1) \div C_o].100$ (Eq. 2)

Where: C_o = initial conductivity of the coenzyme solution (µS/cm); C_1 = conductivity of the supernatant after immobilization.

RESULTS AND DISCUSSION

Immobilization of coenzymes

The variation of the absorbance as a function of the coenzyme concentration is shown in Figures 1 and 2.

The minimum linear square regression equations are

$Y^a = 674x10^{-5} + 0.181x10^{-5}.A$ (r = 0.998) (Eq. 3)

$Y^b = 598x10^{-5} + 0.138x10^{-5}.B$ (r = 0.994) (Eq. 4)

$Y^c = 439x10^{-5} + 0.0560x10^{-5}.C$ (r = 0.998) (Eq. 5)

$Y^d = 767x10^{-5} + 0.165x10^{-5}.D$ (r = 0.990) (Eq. 6)

$Y^e = 484x10^{-5} + 0.141x10^{-5}.E$ (r = 0.991) (Eq. 7)

$Y^f = 493x10^{-5} + 0.0568x10^{-5}.F$ (r = 0.993) (Eq.8)

Where: Y^a, Y^b, Y^d, and Y^e refer to the absorbance read at 260 nm; Y^c and Y^f refer to the absorbance read at 340 nm; A = concentration of NAD; B = concentration of NADH; C = concentration of NADH read at 340 nm; D = concentration of NADP; E = concentration of NADPH; and F = concentration of NADPH read at 340 nm.

The values of the derivatives of equations 3-8 are presented in Table 1.

Table 1: Derivatives of the equations correlating the changes in absorbance as a function of coenzyme concentration (expressed as µMx10^{-5}).

Wavelength	Derivative	NAD	NADH	NADP	NADPH
260 nm	(dY^a/dA)	0.181	-	-	-
	(dY^b/dB)	-	0.138	-	-
	(dY^d/dD)	-	-	0.165	-
	(dY^e/dE)	-	-	-	0.141
340 nm	(dY^c/dC)	-	0.056	-	-
	(dY^f/dF)	-	-	-	0.0568

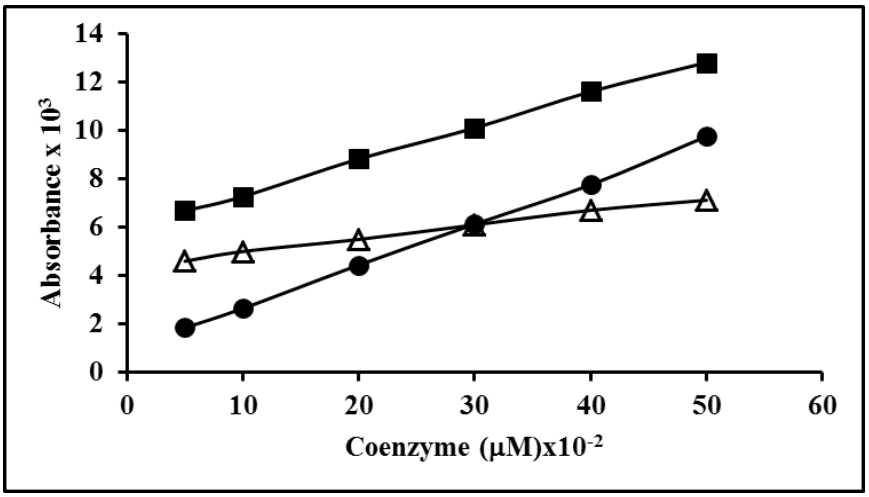

Figure 1: Variation of absorbance, measured at 260 nm and 340 nm, versus coenzyme concentration. Symbols: λ = 260 nm: NAD (●), NADH (■); λ = 340 nm: NADH (Δ).

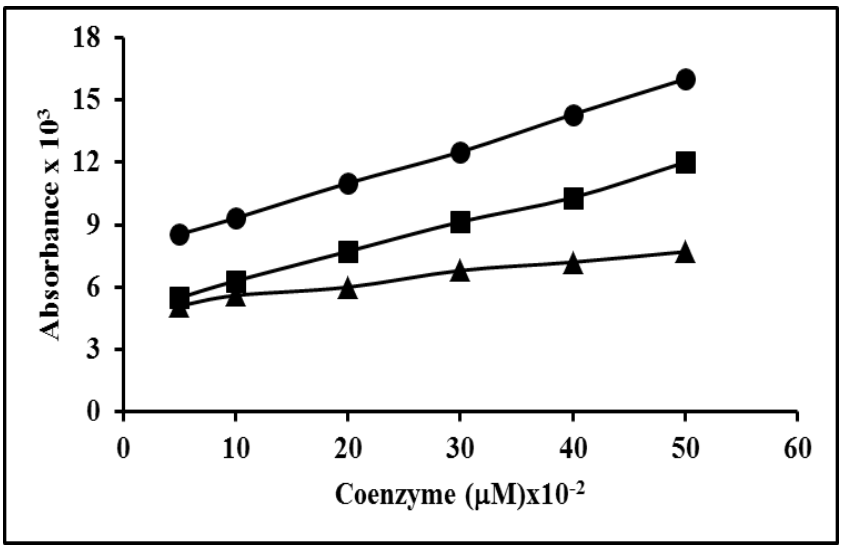

Figure 2: Variation of absorbance, measured at 260 nm and 340 nm, versus coenzyme concentration. Symbols: λ = 260 nm: NADP (●), NADPH (■); λ = 340 nm: NADPH (▲).

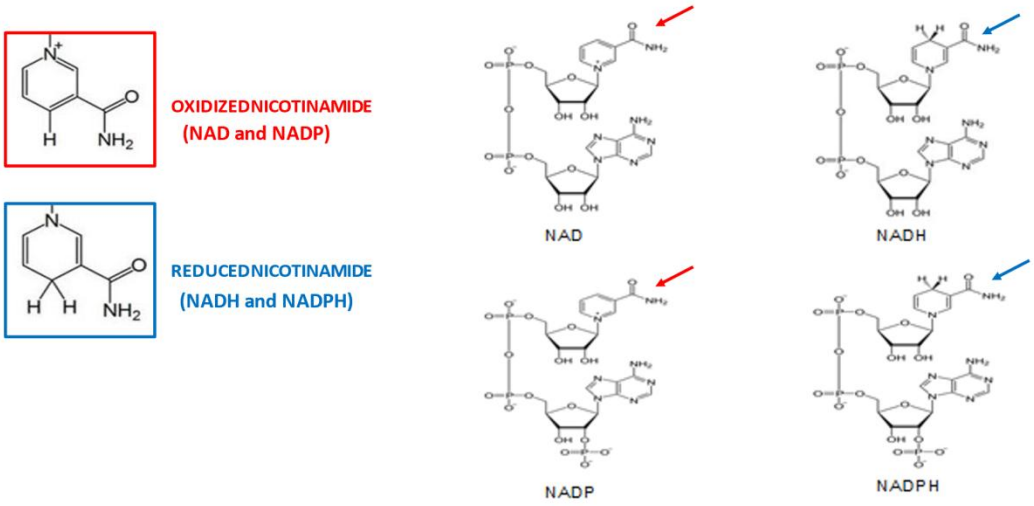

FIGURE 3. Molecular structures of nicotinamide adenine dinucleotides esterified (NADP) or not (NAD) with phosphate.

The nicotinamide adenine dinucleotides studied differed in the redox form of the nicotinamide group (reduced or oxidized) and in the presence or not of phosphate in the C2 of the ribose ring of the adenine mononucleotide group (Figure 3). By comparing the derivative values [$(dY^b/dB) \div (dY^a/dA) = 0.76$] and [$(dY^e/dE) \div (dY^d/dD) = 0.85$], we noted that the oxidized nicotinamide group was responsible for 24% and 15% of the total absorption (at λ = 260 nm) of NAD and NADP, respectively. However, either at λ = 340 nm [$(dY^c/dC) \div (dY^f/dF) = 0.99$] or at λ = 260 nm [$[(dY^b/dB) \div (dY^e/dE) = 0.98$], the reduced form of nicotinamide practically did not affect the total absorption, i.e., the reduced nicotinamide group does not absorb NAD and NADP at 260 nm and 340 nm. The NADH/NAD or NADPH/NADP ratio can be used as criterion to verify the balance of a coupled enzyme system, insofar as the equilibrium NAD ⇆ NADH or NADP ⇆ NADPH remains invariable.

The coenzymes adsorbed on anionic resins presented an immobilization efficiency (IE) higher than 95% regardless of the pH of the immobilization medium (7.5 or 8.5) (Table 2). Moreover, 100 mg of resin adsorbed 22 μM of any type of coenzyme. Thus, the immobilization of coenzymes on anionic resins is a promising method to become usable either in industry bioreactors or in enzyme assay kits.[6] It was shown that NADPH may be immobilized in polyvinyl alcohol gel beads, which can be used in the synthesis of chiral alcohols from ketones by alcohol dehydrogenase in presence of 2-propanol as co-substrate.[7]

Table 2: Immobilization efficiency of coenzymes adsorbed on 100 mg of 100 mesh-anionic resins (types 1X2 and 1X8) at pH 7.5 and 8.5.

Wavelength (nm)	Coenzyme (name)	Anionic resin[5]	
		1X2	1X8
260	NAD	(96/96) [a]	(96/97)
	NADP	(100/100)	(100/98)
	NADH	(100/100)	(100/97)
	NADPH	(100/100)	(100/98)
340	NADH	(100/100)	(100/100)
	NADPH	(100/100)	(100/100)

[a]The first and second values refer to pH 7.5 and 8.5, respectively.

Desorption tests

The aim of these tests was to determine the percentage of coenzymes detached from the support if the coenzyme-resin complex was used in a continuous process during a long duration (over 50 h). The data is presented in Table 3.

Table 3: Percentage of coenzyme desorption from the anion exchange resin.

Coenzymes (names)	Resins (types)	Desorption (%)	
		pH 6.0	pH 9.0
NAD	1X2	8.1	10.7
	1X8	8.6	11.4
NADP	1X2	10.5	9.2
	1X8	11.8	10.6
NADH	1X2	12.5	13.9
	1X8	11.2	13.8
NADPH	1X2	11.0	14.8
	1X8	12.2	14.5

Table 3 shows that desorption was higher at pH 9.0 than at pH 6.0. Moreover, the reduced forms of nicotinamide (NADH and NADPH) detached more than the oxidized ones for both pHs. Probably, the reduced form was not fully compatible with the anionic resin for presenting only negative phosphate groups (Figure 3). Thus, NAD and NADP may be adsorbed on anionic resin, whereas NADH and NADPH would be adsorbed in cationic resin. Anyway, immobilization by adsorption of coenzymes was remarkable, allowing for long-duration continuous processes.

Evaluation of immobilization by conductivity

The conductometer was calibrated by measuring the conductivity of the KCl solution at concentrations of 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, and 10 mM (Figure 4). Calibration curves for correlating conductivity and coenzyme concentration were also performed (Figures 5 and 6).

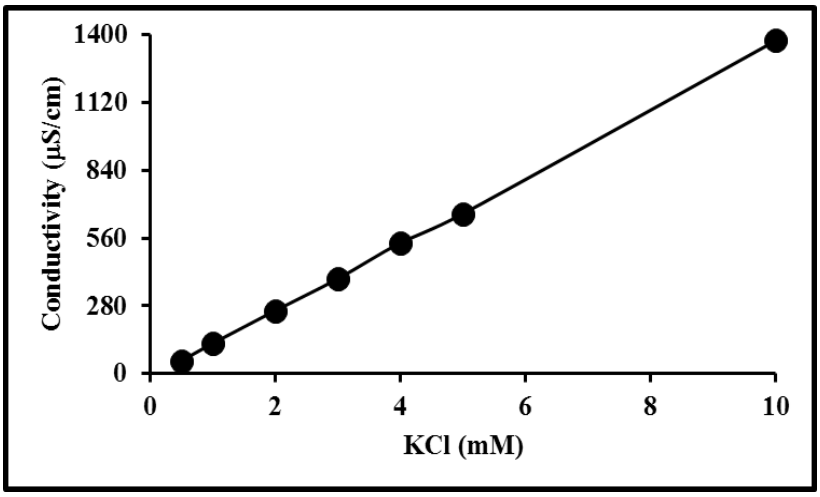

Figure 4: Variation of conductivity against KCl concentrations.

The variation coefficient of the method is 1.06% and the minimum square linear regression equation is:

$y = 138.6x - 18.42$ ($r=0.99998$) (Eq.9)

Where: y = conductivity (µS/cm) and x = KCl concentration (mM).

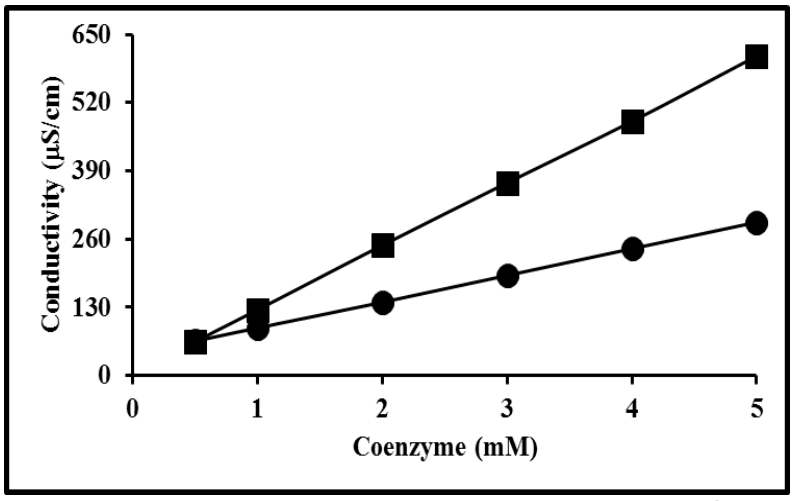

Figure 5: Variation of conductivity as a function of NAD(●) and NADH (■) concentrations.

The minimum square linear regression equations are:

$y_1 = 50.32x_1 + 40.21$ (r= 0.9994) (Eq. 10)

$y_2 = 120.51x_2 + 5.41$ (r= 0.9996) (Eq. 11)

Where: $y_1$and y_2 are the conductivities (µS/cm) of NAD and NADH, respectively; and x_1 and x_2 are the concentrations (mM) of NAD and NADH, respectively.

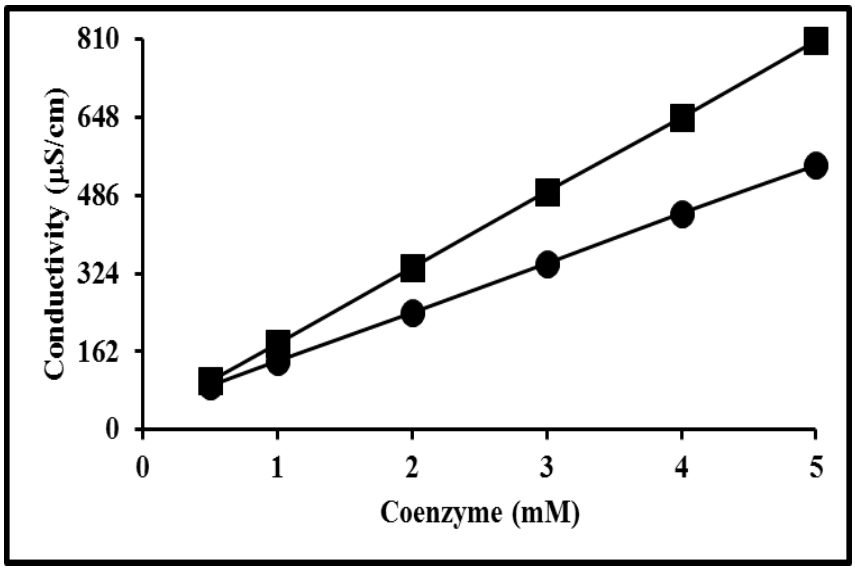

Figure 6: Variation of conductivity as a function of NADP (●) and NADPH (■) concentrations.

The minimum square linear regression equations are:

$y_3 = 101.6x_3 + 40.9$ (r= 0.9998) (Eq. 12)

$y_4 = 156.47x_4 + 23.09$ (r= 0.9995) (Eq. 13)

Where: y_3 and y_4 are the conductivities (µS/cm) of NADP and NADPH, respectively; and x_3 and x_4 are the concentrations (mM) of NADP and NADPH, respectively.

The differences on conductivity among the coenzyme solutions are caused by the different number of positive (oxidized nicotinamide group) and negative (phosphate groups) charges present in their structures (Figure 3). From Table 4, we note that the presence of positive charge in NAD and NADP causes a conductivity decrease of 58% and 35% regarding the conductivity of NADH and NADPH, respectively.

Table 4: Comparison of conductivities of the aqueous solutions of coenzymes.

Coenzyme (name)	Charges		Derivatives	
	Negative	Positive	(dy/dx)	Ratio*
NAD	2	1	50.32	
NADH	2	-	120.51	0.42
NADP	4	1	101.6	
NADPH	4	-	156.47	0.65

*Derivative (dy/dx) ratio between the oxidized and reduced forms of the coenzymes.

Enzyme reactions that require coenzymes are frequently carried out in phosphate-buffered solutions. Thus, the conductivity values related to the 1 mM phosphate buffer (pH 6.0, 7.5 or 9.0) are shown in Table 5. These values must be taken into consideration when the conductivity is used as a measurement parameter in coenzyme/resin immobilization or desorption studies. In principle, the extra conductivity (EC) introduced by the ions of the buffer would not be a problem to the procedure, as the conductivity of the various ions present in the buffer are additive.[8] Thus, the EC is simply subtracted from the total conductivity measured. We noted from Table 5 that the pH of the buffer interfered with the conductivity of the solution. Of course, the accuracy of the conductimetric measurement increases as the buffer is more diluted. However, there is a limit for buffer dilution imposed by the enzyme activity, as any enzyme requires a minimal buffer strength for promoting catalysis.[9][10][11]

Table 5: Conductivity values attained for 1 mM phosphate buffer at different pHs.

pH	Conductivity (µS/cm)
6.0	114.70
7.5	529.40
9.0	623.40

Table 6 shows the results regarding the desorption of coenzymes from the resin using the conductance method.

Table 6: Desorption of coenzymes from anion exchange resins, Measured by conductimetry.

Coenzyme (name)	Resin (type)	Desorption (%)	
		pH 6.0	pH 9.0
NAD	1X2	4.81	9.68
	1X8	9.07	13.8
NADH	1X2	11.7	14.7
	1X8	9.93	14.4
NADP	1X2	9.85	9.76

	1X8	12.0	15.0
NADPH	1X2	11.0	14.0
	1X8	11.9	14.5

Comparing the data presented in Tables 3 and 6, we can note that the percentage of desorption calculated by either spectrophotometry or conductimetry was quite similar. Thus, we may use these methods indistinctly for evaluating the leakage of coenzymes from a bioreactor operated at continuous mode, in which adapting a micro-flow meter capable to measure the absorbance or the conductivity of the outlet solution at the exit of the bioreactor would be enough.

CONCLUSION

The data presented allowed to conclude that the adsorption of coenzymes on anionic resins (types 1X2 and 1X8) is efficient (immobilization efficiency of over 95%). Furthermore, the desorption of coenzymes from the resins was lower than 15%, regardless of pH. The use of both spectrophotometry and conductimetry for evaluating coenzyme leakage might be useful in association with other parameters to control the performance of a continuous bioconversion process.

REFERENCES

1. Vitolo M. Overview on glucose oxidase. World Journal of Pharmaceutical Research, 2021; 10(14): 130-155.
2. Yoriyaz EJ, Vitolo M. Reduction of prochiral ketones by NAD(H)-dependent alcohol dehydrogenase in membrane reactor. Athens Journal of Sciences, 2015; 2(3): 161-176.
3. Vitolo M. Overview on immobilization. World Journal of Pharmaceutical Research, 2021; 10(11): 2121-2145.
4. Tomotani EJ, Vitolo M. Screening of Dowex® anion exchange resins for invertase immobilization. Applied Biochemistry and Biotechnology, 2004; 113: 145-159.
5. Vitolo M. Adsorption of invertase on anionic resin. European Journal of Pharmaceutical and Medical Research, 2020; 7: 113-117.
6. Lin W, Wang P. Cofactor regeneration for sustainable enzymatic biosynthesis. Biotechnology Advances, 2007; 25: 369-384.
7. De Temiño DMR, Hartmeier W, Ansorge SMB. Entrapment of the alcohol dehydrogenase from *Lactobacillus kefir* in polyvinyl alcohol for the synthesis of chiral

hydrophobic alcohols in organic solvents. Enzyme Microbial and technology, 2005; 36: 3-9.

8. Ewing GW. Instrumental Methods of chemical Analysis. McGraw-Hill, Inc. New York, 1969; 3.
9. Vitolo M. Some notes on the characteristics of water. World Journal of Pharmacy and Pharmaceutical Sciences, 2021; 10(6): 109-133.
10. Vitolo M. Brief review on enzyme activity. World Journal of Pharmaceutical Research, 2020; 9: 60-76.
11. Taraboulsi FA, Yoriyas EJ, Vitolo M. Conductance of β-nicotinamide adenine coenzymes in aqueous solution: application on the evaluation of desorption of coenzymes immobilized on resins. PHARMANEST, 2014; 5: 1866-1873.