Reduction of Prochiralic Ketones by NAD(H)-dependent Alcohol Dehydrogenase in Membrane Reactor

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Chiral alcohols are intermediates of organic synthesis protocols for attaining flavors, pheromone, phytohormone and therapeutic molecules. This work dealt with the conversion of acetophenone and hexanone into1-phenyl ethanol and 2-hexanol, respectively, by NAD(H)-dependent alcohol dehydrogenase (ADH) in a membrane reactor (MR) operated in a continuous regimen, there being at the bottom a nanofiltration membrane (MWCO 500Da). The conversions, in which the ethanol was employed as a co-substrate, were carried out at 100rpm, a feeding rate of 5mL/h, ADH (150U), temperature (30ºC), pH (8.8), initial substrate concentration (30mM) and β-NAD/β-NADH (0.9mM). The ADH activity as well as the factors affecting it (temperature and initial substrate concentration) was measured through the consumption or formation of β-NADH at 340nm. The optimum temperature for 5min of reaction was 45oC. The Michaelis-Menten constant related to the action of ADH on NADH, acetophenone and 2-hexanone were $(K_M)NADH = 0.177mM$, $(K_M)acetophenone = 1.04mM$ and $(K_M)2hexanone = 1.44mM$. Along the reaction, the NAD/NADH regeneration occurred and the membrane used retained both the ADH and the coenzymes (NAD; NADH) inside the reactor. Finally, the acetophenone/1-phenylethanol and 2-hexanone/2-hexanol conversions through the MR were 60% and 30%, respectively.
Keywords: *Membrane reactor, Alcohol dehydrogenase, 2-hexanol, 1-phenyl ethanol*

Introduction

During the last few decades, the interest in using biocatalysts has been growing. The reason is that they convert a specific molecule (the substrate) into a desired bio-product at a high rate under mild conditions, often generating, non-toxic effluents into the environment. The possibility of carrying out coupled reactions - two or more enzymes acting in sequence, two enzymes acting in opposite directions (Andreotti et al., 2010) and one coenzyme-dependent enzyme acting in opposite directions according to the pH of the reaction medium (Riebel, 2003) – constitutes a valuable approach to the enzyme technology.

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Chiral alcohols are intermediates of organic synthesis protocols for attaining among other flavors, pheromone, phytohormone and therapeutic molecules (Goldberg, 2007).

The reversible conversion of ketones/aldehydes into primary and/or secondary alcohols can be achieved by using the yeast alcohol dehydrogenase (ADH), an enzyme which requires the NAD/NADH coenzymes as co-substrates. However, the coenzymes are expensive substances which must be regenerated during the catalysis, if an effective use of ADH is envisaged (Lutz et al., 2006). Furthermore, the coenzyme regeneration avoids the eventual inhibition caused by the product on its actuation as a co-substrate (Van der Donk and Zhao, 2003). An alternative would be the use of a whole yeast cell in order to take advantage of the natural presence of coenzymes and ADH inside the cell. Nevertheless, the catalytic performance of an intact cell is quite low due mainly to the hindrance posed by both the cell wall and the plasmatic membrane to the in/out diffusion of substrates and products. The whole-cell catalyst leads to the occurrence of undesirable side reactions and the attainment of low volumetric productivity as well. The approaches envisaged for regenerating the coenzyme would thus be either by coupling two enzyme catalyses (one NAD and the other NADH-dependent, for instance) or by one enzyme (such as yeast ADH) which has its oxidation (pH$\leq$ 7.0) and reduction (pH$\geq$ 8.0) capabilities exchanged through the pH variation of the reaction medium (Wildeman at al., 2007; Findrik et al., 2007).

The type of reactor to be chosen must fulfill two pre-conditions which are both retaining the coenzyme molecule (MW near 700Da) and allowing its prompt regeneration.

Among all kinds of reactors available – mainly a packed- or fluidized-bed reactor in which the coenzyme might be obligatorily in the immobilized form -, the membrane reactor (MR) seems to be the most adequate because it can be operated both in a discontinuous or a continuous mode as well as with the coenzyme in a soluble or an immobilized form (Curcio et al., 2002).

The MR is a variant of the continuous stirred tank reactor (CSTR) in which an ultra (UF) (pore diameter: 0.001 a 0.1μ) or nanofiltration (NF) (pore < 2nm) membrane was coupled. It can be designed either by connecting in series the CSTR to a membrane container (two-module-MR) or by adapting the membrane to the bottom of the CSTR as in a stirred ultrafiltration cell (one-module-MR). Although the two-module-MR is the preferred one – in the literature, there are several processes described in which L-amino acids, alcohols and R-hydroxyacids are attained by using NAD(P)H-dependent dehydrogenases (Wildeman et al., 2007; Findrik et al., 2007) –, the one-module-MR has some favorable operational characteristics such as a homogeneous catalysis, the use of two or more enzymes (multienzymatic reactions), a high activity per unit of volume and the absence of diffusion effects on the overall transit of substances in/out of the reactor (Tomotani and Vitolo, 2007). The employment of membrane reactors since 2000 has led to the development of several processes which among others are: the hydrolysis of casein and hemoglobin (Curcio et al., 2002); the hydrolysis of sucrose (Yoriyaz

and Vitolo, 2014); the synthesis of cyclodextrins (Slominska et al., 2002), fructooligosaccharydes (Sheu at al., 2002), cathecol (Boshoff et al., 2003) and esters (Lozano et al., 2004); a wastewater and industry effluent treatment (Chen, 2008; Martin-Pascual et al., 2015; Ghalavand et al., 2015).

In this work, the feasibility of using NAD(H)-dependent alcohol dehydrogenase (ADH), attained from *Saccharomyces cerevisiae*, as a means of simultaneous regeneration of coenzymes (β-NAD ↔ β-NADH) as well as obtaining chiral alcohols (1-phenylethanol and 2-hexanol) from prochiral ketones (acetophenone and 2-hexanone) by using a one-module membrane reactor (MR) coupled with a NF-membrane (MWCO 500Da) were studied.

Materials and Methods

Materials

Alcohol dehydrogenase (ADH – E.C.1.1.1.11) from *Saccharomyces cerevisiae* and the coenzymes (β-NAD and β-NADH) were purchased from Sigma, St. Louis, USA. The NF-membrane (MWCO 500Da) was kindly donated by Bioengineering® AG. All of the other chemicals used were also of the highest analytical grade.

Methods

Standard Assay for Measuring ADH Activity

ADH activity was assayed spectrophotometrically at $45^{\circ}C$ by measuring the change in absorbance of NADH (formed or consumed, respectively, from the oxidation or reduction reaction) at 340nm using a Beckman DU 640 spectrometer equipped with a Peltier effect-controlled temperature cuvette holder (Taraboulsi-Jr. et al., 2014).

The standard assay for the coenzyme oxidation reaction was carried by adding 10µL of ADH (0.75U/mL), 440 µL of 10mM phosphate buffer (pH 7.0), 500 µL of 10mM 2-hexanone or acetophenone, 50 µL of 2mM β-NADH and 10 µL of the mixture [0.1% (w/v) of BSA and 10% (v/v) of DMSO]. In the blank tube, all reagents cited, except for the ADH solution was introduced.

The standard assay for the coenzyme reduction reaction was carried by adding 50µL of ADH (0.75U/mL), 50 µL of 10mM phosphate buffer (pH 8.8), 500 µL of 10mM 2-hexanol or 1-phenylethanol, 400 µL of 2mM β-NAD and 50 µL of the mixture [0.1% (w/v) of BSA and 10% (v/v) of DMSO]. In the blank tube, all reagents cited, except the ADH solution was introduced.

The enzyme activity, expressed as U/mL, was calculated through the equation:

$$(U/mL)_{ADH} = V.f.[(\triangle Abs_{340}/min)_{test} - (\triangle Abs_{340}/min)_{blank}] \div [a_m.v] \qquad (Eq.\ 1)$$

Where: V = reaction volume; f= dilution factor; $(\triangle Abs_{340}/min)_{test}$ = absorbance variation of the test tube during 1min reaction; $(\triangle Abs_{340}/min)_{blank}$

= absorbance variation of the blank tube during 1min reaction; a_m = 6.22mM^{-1}.cm^{-1}; v = volume of ADH solution.

One unit of ADH represented 1μmol of β-NADH produced or consumed per minute at 45°C, on a basis of an absorption coefficient of 6.22 mM^{-1}.cm^{-1} for β-NADH at 340nm.

Characterization of Alcohol Dehydrogenase

The pH, temperature and substrate concentration of the standard reaction test related to ADH were changed one by one at the intervals cited in the following sections.

Effect of pH and the Concentration of the Buffer on ADH Activity and Stability

The effect of pH on the activity and stability of ADH was always determined at 45°C by mixing the enzyme with buffer solutions at a fixed pH (7.0, 7.5, or 8.8) (Andreotti et al., 2010). The buffer used was 10mM or 50mM phosphate buffer for all pH studied. The stability against pH was evaluated by measuring the residual activity of ADH in samples taken at each 2h up to 24h of enzyme-buffer contact at 45°C.

Effect of Temperature on Activity and Stability

The effect of the temperature on enzyme activity was evaluated by varying the temperature of the standard ADH assay between 25°C and 50°C.The evaluation of the ADH stability against temperatures of 30°, 35°, 40°, 45° and 50°C was carried out by leaving a 10mM phosphate buffer (pH 7.0) solution of ADH (0.75U/mL) at each temperature for a time interval of 10-1440min. The residual ADH activity was measured as described above.

Effect of the Initial Substrate Concentration

The ADH activity was measured against an initial concentration of acetophenone, 2-hexanone, ethanol or β-NADH. The correspondent kinetic constants were calculated by applying the conventional Lineweaver-Burk method.

Batch Bioconversion

In a 100mL-flask, 25mL of 10mM acetophenone or 2-hexanone, 5mL of 15mM β-NADH, 50mL of 10mM phosphate buffer (pH 7.0) and 2mL of ADH buffered solution (340U) were introduced. The reaction was carried out at 30°C for 30min. The 1-phenyl ethanol or 2-hexanol formed was measured by the method of potassium dichromate (Isarankura-Na-Ayudhya, 2007). Aliquots were removed every 5min for measuring the alcohol formed.

Continuous Bioconversion

In a 10mL-membrane reactor coupled with a 500Da NF-membrane, 10mL of aqueous solution containing 1mM β-NADH and ADH (150U) was introduced. The reactor was fed with the buffered solution (pH 7.0) of 10mM

substrate (acetophenone or 2-hexanone) at a feeding rate of 2mL/h. The overall duration of the continuous feeding was 96h at 30^oC and 100rpm. Samples were taken every 2h for analytical purposes. The feeding of the reactor along the reaction was made by alternating the ketone solution with a 10mM ethanol buffered solution (pH 8.8), in order to promote β-NADH/β-NAD recycling along the overall continuous process. The membrane reactor employed was purchased from Bioengineering AG (Wald, Germany). It is a 316-L stainless steel cylinder, whose bottom has an inlet and an outlet for the external water bath for the temperature control. The reactor employs a 63mm diameter UF- or NF-membrane, it can be sterilized as well (autoclave up to 134^oC for 30min) and resists high temperatures (up to 150^oC) and corrosion by most substances (except strong acids, pH <1.0; and alkalies, pH > 12.0). It supports pressure up to 6bar and can be coupled to a dosing pump, a pressure probe, a sterile filter and a bubble trap.

The membrane integrity along the reaction was monitored by measuring the protein and coenzyme presence in the outlet solution (Taraboulsi-Jr. et al., 2014). The absence of protein and coenzyme was taken as the criterion for membrane integrity.

Analytical Techniques
Measurement of Carbonyl Compounds with 2,4-dinitro-phenylhydrazine

The determination was based on the approach proposed by Purnendu et al. (1994). The reagent solution was prepared by dissolving 0.1g of 2,4-dinitro-phenylhydrazine and 0.5 mL of concentrated HCl in 100mL of acetic acid. The determination of acetaldehyde, 2-hexanone or acetophenone was carried out by mixing 1mL of the sample with 5mL of reagent and leaving the mixture at room temperature for 1h. The intensity of the color formed was read by a spectrophotometer at 480nm. The absorbance was compared to the correspondent standard curve made with acetaldehyde, 2-hexanone or acetophenone (Figure 4).

Measurement of Alcohols with Potassium Dichromate

The determination was based on the method proposed by Isarankura-Na-Ayudhya, (2007). The reagent solution was prepared by dissolving 7.4g of potassium dichromate in 190mL of distilled water followed by the addition of 60mL 0.5M sulfuric acid solution. The reagent was left resting for 48h before use. The determination of ethanol, 2-hexanol or 1-phenylethanol was carried out by mixing 1mL of the sample with 4mL of distilled water and 1mL of reagent and leaving the mixture at room temperature for 10min. The intensity of the color formed was read by spectrophotometer at 590nm. The absorbance was compared to the correspondent standard curve made with ethanol, 2-hexanol or 1-phenylethanol (Figure 4).

<u>Protein Determination</u>

Protein was determined based on the difference between UV absorbance measured at 215nm and 225nm, using bovine serum albumin (BSA, from Sigma, St. Louis, USA) as a standard (Tomotani and Vitolo, 2007).

<u>Cofactor Determination</u>

Both cofactors, β-NAD and β-NADH, were determined spectrophotometrically at 260 and 340nm, respectively (Taraboulsi-Jr. et al., 2014).

Results

The ADH activity was measured against both acetophenone [0.15(ABS_{340nm})/min] and 2-hexanone [0.11(ABS_{340nm})/min] (Figure 1). The ADH activity was also measured against ethanol [0.090(ABS_{340nm})/min] because it was used as an auxiliary substrate for converting the cited ketones into the respective chiralic alcohols through the membrane reactor.

Figure 1. *Alcohol Dehydrogenase Activity Measured by using Ethanol (•), Acetophenone (■) and 2-hexanone (Δ) as Substrates. The Least-square Linear Regression Equations for Ethanol, Acetophenone and 2-hexanone were, Respectively, $Y_E = 9x10^{-2}.t + 0.72$ (r = 0.997), $Y_A = - 0.15.t + 0.98$ (r = 0.994) and $Y_H = - 0.11.t + 0.87$ (r = 0.996)*
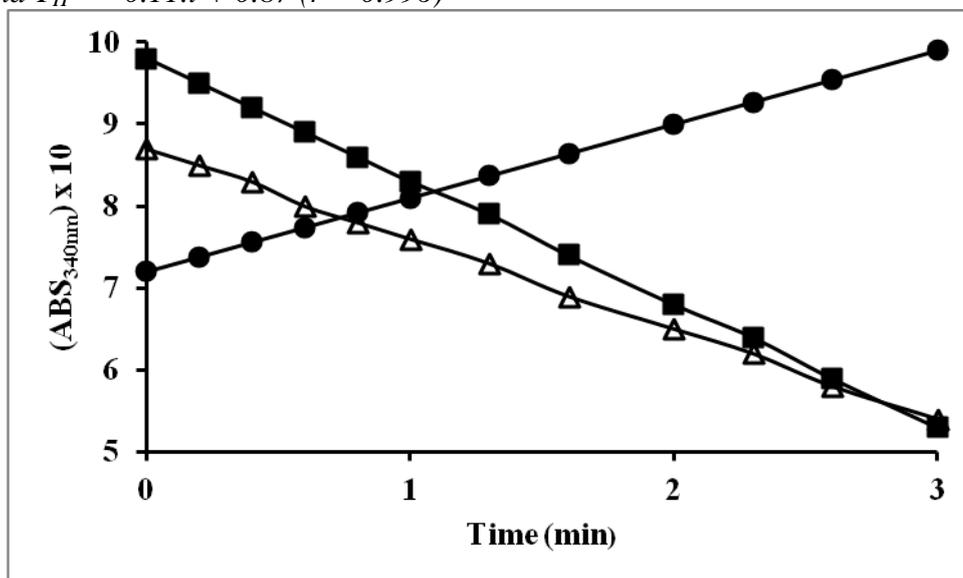
The effect of pH (7.0, 7.5 or 8.8) and the phosphate buffer concentration (10mM or 50mM) on the ADH activity are shown in Table 1.

Table 1. *Effect of pH and Phosphate Buffer Concentration on the Alcohol Dehydrogenase Activity (U/min) Measured against Acetophenone (ADH$_A$) and 2-hexanone (ADH$_H$) as Substrates*

	ADH activity (U/min)	
	Phosphate buffer	
pH	10 mM	50 mM
7.0	*645/550	807/686
7.5	641/545	844/717
8.8	502/427	509/433

*First and second values refer to the ADH$_A$ and ADH$_H$, respectively

Considering that the ADH activity has high sensitivity to the pH variation of the reaction medium – at $6.6 \leq pH \leq 7.0$, it catalyses the oxidation of the coenzyme and reduction of the carbonyl compound whereas at $pH > 7.0$, it promotes the reduction of coenzyme and the oxidation of the alcohol – there was no need to evaluate the activity against a larger pH interval. Thereby, the pH was set at 7.0 and 7.5 and the temperature of the reaction varied between $25^{\circ}C$ and $50^{\circ}C$ (Figure 2).

Figure 2. *Variation of Alcohol Dehydrogenase Activity against the Temperature using Acetophenone [pH 7.0 (●) and pH 7.5 (o)] and 2-hexanone [pH 7.0 (■) and 7.5 (□) as the Substrates. The Overall Reaction Time was Equal to 5min*
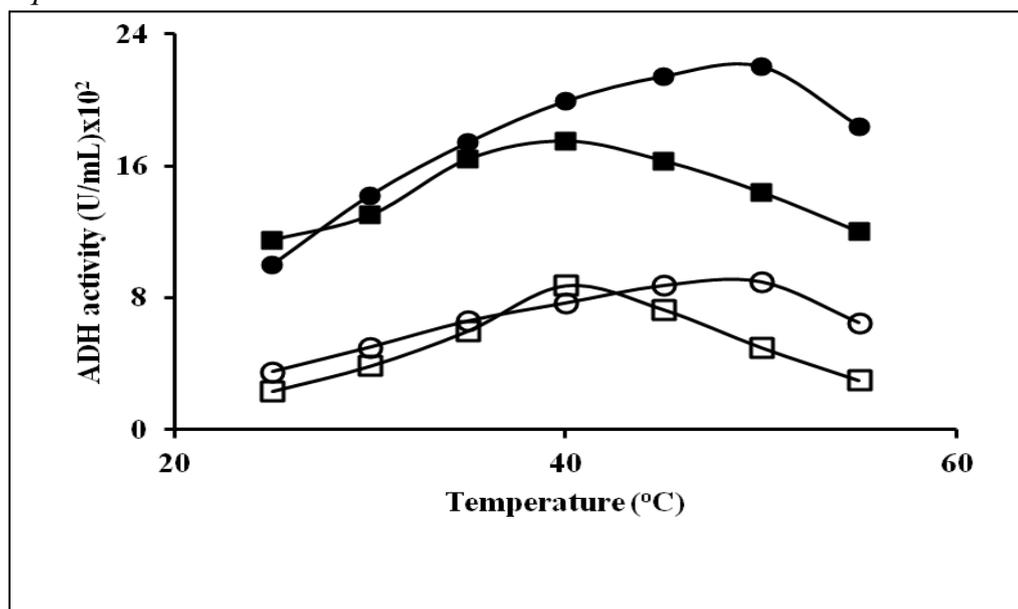
The stability of ADH for 24h was made leaving the buffered enzyme solution (1000 U/mL) at pH 7.0 and temperatures of $40^{\circ}C$ and $50^{\circ}C$. The residual ADH activity was measured against 2-hexanone and acetophenone as substrates (Figure 3).

Figure 3. *Variation of the Residual Activity of ADH Dissolved in 50mM Phosphate Buffer (pH 7.0), the Solution being maintained at 40°C and 50°C for 24h. The Enzyme Activity was Measured using 2-hexanone [40°C (●) and 50°C (■)] and Acetophenone [40°C (△) and 50°C (♦)]*
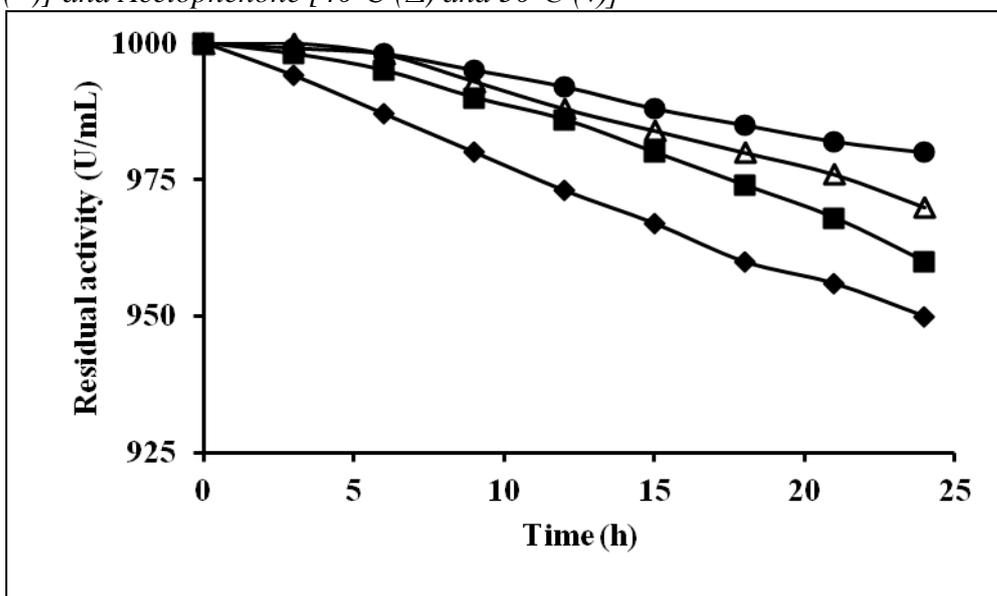
The effect of initial concentrations of β-NADH, 2-hexanone and acetophenone on ADH activity were determined (Table 2) and the respective kinetic constants (K_M and V_{max}) calculated through the Lineweaver-Burk's method.

Table 2 *ADH Activity against Initial Concentrations of β-NADH, Acetophenone and 2-hexanone*

β-NADH (mM)x10^3	v_{nadh} (U)x10^3	Acetophenone (mM)x10^2	$v_{acetophenone}$ (U)x10^4	2-hexanone (mM)x10^2	$v_{2-hexanone}$ (U)x10^4
25	86,3	15	143	20	132
50	154	30	329	40	235
65	187	40	408	50	278
90	235	55	508	75	370
100	252	60	538	85	401
130	295	80	639	100	443
160	331	90	682	130	512
200	370	120	788	160	568
250	408	150	868	200	628

Figure 4 shows the standard curves for measuring the concentration of alcohols (ethanol, 1-phenyl ethanol and 2-hexanol) and ketones (acetophenone and 2-hexanone) by using the reagents potassium dichromate and 2,4-dinitro-phenylhydrazine, respectively. The color intensity of each solution was read

through the spectrophotometer at wavelengths of 590nm (alcohols) and 480nm (ketones).

Figure 4. *Standard Curves regarding the Determination of Alcohols [1-Phenylethanol (o), 2-hexanol (□) and Ethanol (Δ)] and Ketones [Acetophenone (•) and 2-hexanone (■)] by using, Respectively, Potassium Dichromate and 2,4-dinitro-phenylhydrazine Reagents, whose Color Intensities of Solutions were Read at Wavelengths of 590nm and 480nm, Respectively*
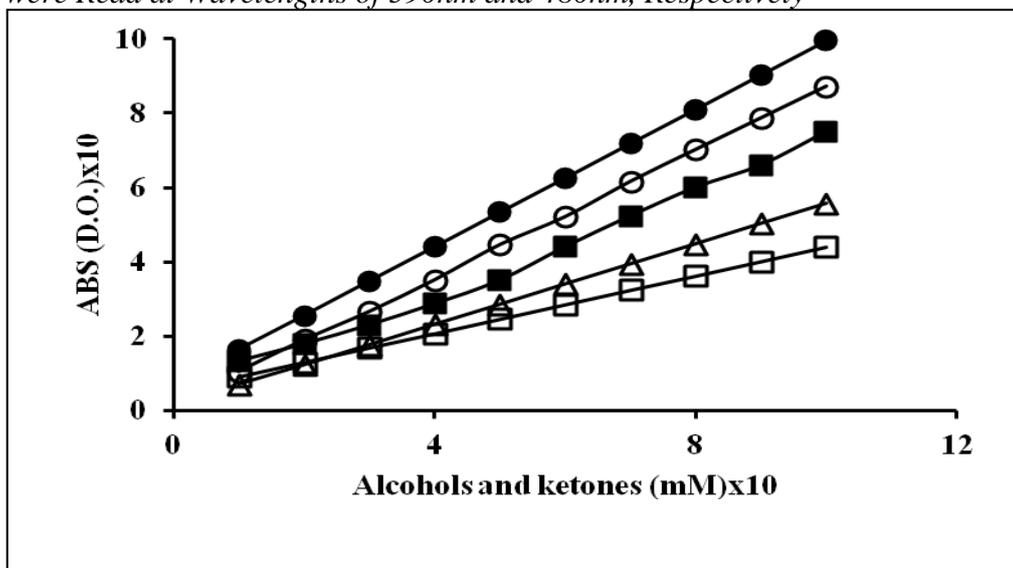
The alcohol (1-phenyl ethanol and 2-hexanol) formations and ketones consumption (acetophenone and 2-hexanone) during the batch process were shown in Figure 5.

Discussion

Figure 1 shows that the ADH yeast has a slight preference for acetophenone over 2-hexanone, insofar as the enzyme activity was about 27% higher for acetophenone than for 2-hexanone.

From Table 1, it can be seen that the concentration of the buffer, that is the ionic strength of the solution, affected the ADH activity. For instance, at pH 7.0, the enzyme activity in a 50mM phosphate buffer was 1.25 times higher than in a 10 mM phosphate buffer either to acetophenone or 2-hexanone used as the substrate. This is the reason by which the use of 50mM phosphate buffer is largely employed for ADH activity measurement (Müller, 2010). Besides, the ADH activity diminished as the pH of the medium increased independently on the type of substrate and buffer concentration. It seemed that the ADH yeast preferred catalyzing the oxidation instead of the reduction of the coenzyme. This result points to the suitability for using ADH yeast on converting pro-chiralic ketones into chiralic alcohols. Setting the buffer concentration at 50mM and using either acetophenone or 2-hexanone as the substrate, it can be

seen that the ADH activity has been reduced about 37% when the pH was varied from 7.0 to 8.8. This result corroborates Zanon et al. (2015), which determined that the yeast ADH activity diminished about 40% as the pH was changed from 7.0 to 9.0.

Figure 5. *Conversion of Acetophenone (o) and 2-hexanone (■) into, Respectively, 1-phenyl ethanol (●) and 2-hexanol (□) in a Batch Process*
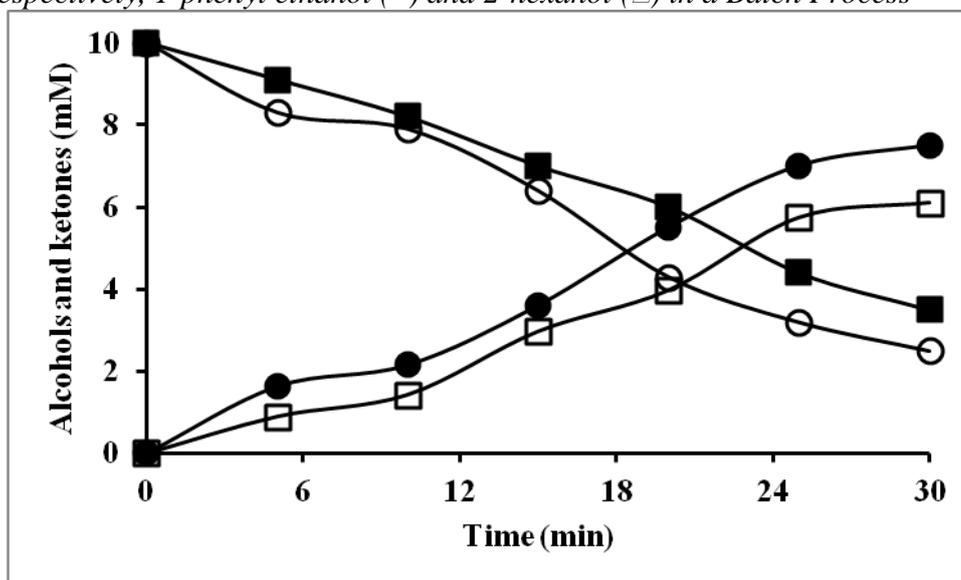

Figure 2 shows that the high ADH activity at pH 7.0 and for 5min of reaction occurred at temperatures of 55°C for acetophenone (2.14×10^{-1} U.mL^{-1}) and 40°C for 2-hexanone (8.40×10^{-2} U.mL^{-1}). So, the optimal temperature under those conditions differs according to the type of prochiral ketone employed as the substrate. Besides, the ADH activity against both ketones diminished at least 50% at pH 7.5. This result, which showed a clear interaction of variables (pH and temperature) on ADH activity, is relatively often in enzyme literature (Fullbrook, 1996). By applying the Arrhenius' method on the data presented in Figure 2, the respective activation energy (E_a) to the reduction of acetophenone by ADH at pH 7.0 ($E_a = 2.5$kcal/mol) and pH 7.5 ($E_a = 3.8$kcal/mol) were calculated. The E_a regarding 2-hexanone at pH 7.0 and 7.5 were 3.3kcal/mol and 3.7kcal/mol, respectively. Thereby, in both cases, carrying out the ketone reduction at pH 7.0 is advisable.

After leaving the alcohol dehydrogenase for 24h at 40°C and 50°C, it was noted that its activity measured against acetophenone and 2-hexanone diminished along the time (Figure 3). By correlating the logarithm of the residual ADH activity with time, the thermal denaturing constant (k_d) regarding both ketones, whose values are presented in Table 3, were calculated. Through the integrated form of Arrhenius' equation (Purich, 2010), the denaturing activation energy of alcohol dehydrogenase against acetophenone ($E'_a = 12$ kcal/mol) and 2-hexanone ($E'_a = 9$ kcal/mol) used as substrates were calculated. Clearly, the structural modification suffered by the ADH molecule

due to the temperature's increasing reflected more intensely on its capacity for reducing 2-hexanone than acetophenone. In other words, to reduce the ADH capability on reducing 2-hexanone to 2-hexanol would require 25% less energy than for reducing acetophenone to 1-phenylethanol.

Table 3. *Alcohol Dehydrogenase Thermal Denaturing Constant (k_d) Related to Ketones Reduction into their Correspondent Alcohols*

KETONE	k_d	
(Name)	40°C	50°C
Acetophenone	-0.0016	-0.0029
2-hexanone	-0.0012	-0.0019

By applying the conventional Lineweaver-Burk's method (Purich, 2010), data presented in Table 2, the following equations were determined:

$$(1/v_{nadh}) = 0.254. (1/S_{nadh}) + 1.432 \qquad (r = 0.99990) \qquad \text{(Eq. 2)}$$
$$(1/v_{acet}) = 7.40. (1/S_{acet}) + 6.32 \qquad (r = 0.9997) \qquad \text{(Eq. 3)}$$
$$(1/v_{2hex}) = 13.32. (1/S_{2hex}) + 9.27 \qquad (r = 0.99994) \qquad \text{(Eq. 4)}$$

Through the above equations, the kinetic constants for NADH (V_{max} = 0.698 U; K_M = 0.177mM), acetophenone (V_{max} = 0.158U; K_M = 1.17mM) and 2-hexanone (V_{max} = 0.108U; K_M = 1.44mM) were calculated. As K_M for acetophenone is about 19% lower than for 2-hexanone, it can be concluded that the enzyme has more affinity to acetophenone. This result corroborates the well known fact that when an enzyme acts on different substrates, there is always a preference.

Clearly the variation on the concentration of alcohols (ethanol, 1-phenylethanol and 2-hexanol) and ketones (acetophenone and 2-hexanone) from 0.1mM to 1.0mM was adequately determined by using the reagents potassium dichromate and 2,4-dinitro-phenylhydrazine, respectively. According to the data shown in Figure 4, the concentrations of alcohols and ketones varied linearly with the absorbance read at 590nm and 480nm, respectively. The least-square linear regression equations for each compound were:

(ETHANOL): $Y_E = 0.543.X_E + 0.0141$ (r = 0.99995)   (Eq. 5)
(2-HEXANOL): $Y_H = 0.387.X_H + 0.0524$ (r = 0.99997)   (Eq. 6)
(1-PHENYLETHANOL): $Y_p = 0.857.X_p + 0.0145$ (r = 0.9998)   (Eq. 7)
(ACETOPHENONE): $Y_A = 0.924.X_A + 0.071$ (r = 0.99992)   (Eq. 8)
(2-HEXANONE): $Y_{2H} = 0.701.X_{2H} + 0.0296$ (r = 0.9996)   (Eq. 9)

Where Y_E, Y_H, Y_p, Y_A and Y_{2H} are the absorbances and X_E, X_H, X_p, X_A and X_{2H} are the concentrations of ethanol, 2-hexanol, 1-phenylethanol, acetophenone and 2-hexanone, respectively.

By using equations 5-9, the amount formed/consumed of each substrate along the reaction catalyzed by ADH in batch and continuous processes were calculated.

The conversion yields of acetophenone/1-phenylethanol and 2-hexanone/2-hexanol were, respectively, 75% and 65%, when the reactions have been carried out batchwise (Figure 5). Regarding to these conversions it was cited by Hummel (1990) that yields up to 80% would be achieved by using the ADH from *Lactobacillus kefir*.

As borne out previously, trying to carry out the ketone/alcohol reaction in the membrane reactor operated under a continuous mode is quite reasonable.

In a previous work, the possibility of recycling the coenzyme (β-NADH/β-NAD) by employing two enzymes – one catalyzing the reduction of coenzyme (glucose 6-phosphate dehydrogenase) and the other (glutamate dehydrogenase) the reverse reaction was demonstrated (Andreotti et al., 2010). After that, the coenzyme recycling was achieved by using the substrate-coupled procedure catalyzed by the yeast NAD(H)-dependent alcohol dehydrogenase (ADH), since the substrates – ethanol and acetaldehyde, in that study – were alternately fed into the membrane reactor (Tomotani and Vitolo, 2011).

Thereby, the conversion of prochiral ketones (acetophenone and 2-hexanone) into chiral alcohols (1-phenylethanol and 2-hexanol) catalyzed by ADH in the presence of β-NADH using a membrane reactor was made by feeding the reactor alternately with acetophenone or 2-hexanone and ethanol (auxiliary substrate). The main result is presented in Figure 6.

Figure 6. *Profiles on the Continuous Conversion in the Membrane Reactor Fed with 10mM Acetophenone (●) and 10mM 2-hexanone (■), using Ethanol (10mM) as the Co-substrate. Conditions of Reaction: 30°C, Feeding Rate = 2mL/h, UF-membrane = 500Da, 100rpm and 50mM Phosphate Buffer [pH 7.0 (Ketone Solution) and pH 8.8 (Ethanol Solution)]. The Up-directed Arrows Indicate Ethanol Addition, whereas Down-directed Arrows Indicate Addition of Acetophenone or 2-hexanone*
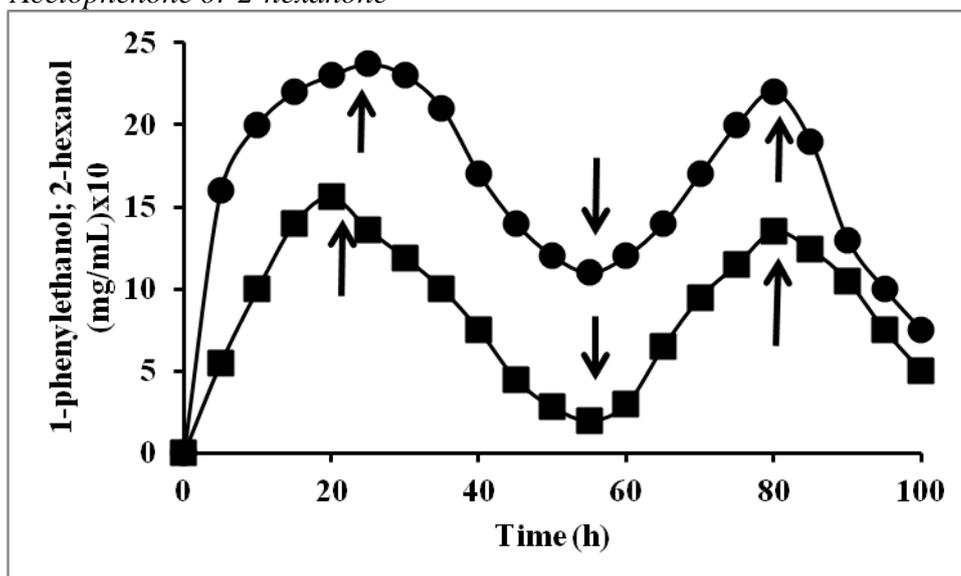
Figure 6 shows clearly that the ketone/alcohol conversion – in the present case acetophenone/1-phenylethanol or 2-hexanone/2-hexanol yield was 60% and 30%, respectively – is a quite possible approach and the process deserves to be improved. When the ethanol is introduced into the reactor, a small volume of the outlet solution will contain both acetaldehyde and chiral alcohol (washed out as ethanol is introduced into the reactor). However, the separation of the aldehyde and the alcohol is easily made by fractionated precipitation, a unit operation well known by chemical engineers and/or chemists.

Conclusions

The data presented allows concluding that the yeast alcohol dehydrogenase (ADH) performed adequately on the reduction of acetophenone and 2-hexanone as well as on the ethanol oxidation. The alcohols and ketones at concentrations of 0.1-1.0mM were also adequately measured by using potassium dichromate and 2,4-dinitro-phenylhydrazine reagents, respectively. The enzyme was sensitive to the ionic strength of the reaction medium, presenting higher activity in 50mM than 10mM phosphate buffer (pH 7.0). The highest ADH activity occurred at $50^{o}C$ (acetophenone) and $40^{o}C$ (2-hexanone). A 50mM-phosphate buffer solution of ADH (pH 7.0) left for 24h at $40^{o}C$ and $50^{o}C$ had its overall activity diminished about 0.14% and 0.25% per hour, respectively. The overall conversions of acetophenone/1-phenylethanol and 2-hexanone/2-hexanol through a batch process were 75% and 65%, respectively. Besides, by alternating the addition of ketone (substrate) with ethanol (co-substrate) into the membrane reactor operated under the continuous mode, the average yields were 60% to acetophenone and 30% to 2-hexanone. Thereby, the continuous process may still be improved.

Acknowledgments

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