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Stabilizing Effects of Deep Eutectic Solvents on Alcohol Dehydrogenase Mediated Systems

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***Abstract*—**This study explored the effects of different organic solvents, temperature, and the amount of glycerol on the alcohol dehydrogenase (ADH)-catalysed stereoselective reduction of different ketones. These conversions were then analyzed by gas chromatography. It was found that when the amount of deep eutectic solvents (DES) increases, it can improve the stereoselectivity of the enzyme although reducing its ability to convert the substrate into the corresponding alcohol. Moreover, glycerol was found to have a strong stabilizing effect on the ADH from *Ralstonia* sp. (*E. coli*/ RasADH). In the case of organic solvents, it was observed that the best conversions into the alcohols were achieved with DMSO and hexane. It was also observed that temperature decreased the ability of the enzyme to convert the substrates into the products and also affected the selectivity. In addition to that, the recycling of DES up to three times gave good conversions and enantiomeric excess results and glycerol showed a positive effect in the stability of various ADHs. Using RasADH, a good conversion and enantiomeric excess into the *S*-alcohol were obtained. It was found that an enhancement of the temperature disabled the stabilizing effect of glycerol and decreased the stereoselectivity of the enzyme. However, for other ADHs a temperature increase had an opposite positive effect, especially with ADH-T from *Thermoanaerobium* sp. One of the objectives of this study was to see the effect of cofactors such as NAD(P) on the biocatlysis activities of ADHs.

***Keywords*—**Alcohol dehydrogenases, DES, gas chromatography, RasADH.

I. Introduction

ALCOHOL dehydrogenases are important enzymes that are widely used to catalyze reversible redox reactions and produce specific alcohols or ketones (Fig. 1). These enzymes are present in many microorganisms such as bacteria and yeast. ADHs are able to catalyze reactions based on NAD and NADH cofactors [1].

The importance of ADHs lay in the fact that they produce enantiopure stereoisomers of chiral alcohols. They can be precursors of important drugs, which are highly demanded by the different pharmaceutical industries [1].

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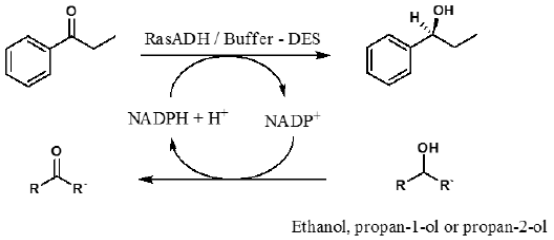

Fig. 1 The reduction of propiophenone to phenyl propanol by Ras/ADH

Since the natural environment of enzymes is water, recent advances in biocatalysis have shown the use of water like solvents with these sensitive enzymes to obtain the desired products while maintaining the enzymes' activity. From this approach, DES have been used in many chemical reactions. DES are composed of a quaternary ammonium salt (like choline chloride) and hydrogen bond donor molecules (HBDs) like polyols [2].

After assessing the use of recombinant whole cells overexpressing oxidoreductases in DES-aqueous mixtures, we investigated in this study the recycling effect of the mono- and bi-phasic systems, which contain the DES focusing on its impact on the ADH stability and selectivity.

This study also investigated the effect of cofactor addition NAD(P) on the conversion and enantioselective activities of ADHs from different microorganisms.

Cofactor recycling is an important area of research because in industrial biocatalytic processes researchers use in a limited manner expensive cofactors. Different techniques of cofactor regeneration have been studied for the possible production of high cofactor amounts [3]. To investigate this point, reactions were prepared and recycled without the addition of extra cofactors like NAD(P).

II. Materials and Methods

The chemicals that were used in this study were bought from Sigma-Aldrich. The enzymes used were obtained by Prof. Wolfgang Kroutil (University of Graz). The ADHs were overexpressed on *E. coli* strain: RasADH from *Ralstonia* sp., SyADH from *Sphingobium yanoikuyae*, TeSADH from *Thermoaerobacter ethanolicus*, ADH-A from *Rhodococcus ruber*, ADH-T from *Thermoanaerobium* sp., and LBADH from *Lactobacillus brevis* [4].

A. The Composition of DES

Three types of DES were used. The first DES (1) was composed of choline chloride (ChCl, 10 g) and ethylene glycol (8 mL). The second DES (2) was made of choline chloride (10 g) and glycerol (10.5 mL), and the third type (3) was composed of choline chloride (10 g) and urea (8.6 g). For the preparation of these solvents, the constituents were mixed and stirred at 60 °C until a clear solution was observed (2 hours) [4].

B. Standard Reaction with Oxidoreductases and DES (80% v/v)

For RasADH, 15 mg of *E. coli*/RasADH was added into an Eppendorf vial, and then, 60 µL of phosphate buffer 100 mM pH 7.5 was added and a solution containing 60 µL of phosphate buffer 100 mM pH 7.5 and $NADP^+$ (10 mM). Then, 480 µL 1:2 (v/v) ChCl:ethylene glycol was added to the mixture as well as propiophenone (3 µL) and propan-2-ol (30 µL). The reaction was left in the shaker (250 rpm) for 24 h at 30 °C. The aqueous phase was extracted by the addition of ethyl acetate (EtOAc, 2 x 500 µL). After separation and drying over sodium sulfate, part of the organic layer (500 µL) was transferred to another Eppendorf vial for derivatization. DMAP (1.0 mg, 8.2 µmol) and acetic anhydride (30 µL, 0.32 mmol) were added and the vial was shaken for 3 h at 30 °C. The reaction was quenched by the addition of water (500 µL) for 30 min. After extraction of the aqueous phase with EtOAc (2 x 500 µL), the combined organic layers were dried over sodium sulfate. The vials with the product solutions were analyzed by using the GC (Agilent CP-ChiraSil-Dex-CB column).

C. Effect of Temperature (45 ºC and 60 ºC) on the Enzymatic Reactions

The reactions were prepared as previously described and left in the shaker (250 rpm) for 24 h at 45 °C. The other set of vials were left in the shaker (250 rpm) for 24 h at 60 °C.

D. Recycling of the Organic Phase

After the extraction of the aqueous phase with ethyl acetate, propiophenone (3 µL), propan-2-ol (30 µL) and the corresponding organic solvent were added to the aqueous phase in the original Eppendorf vial. The reaction was left in the shaker (250 rpm) for 24 h at 30 °C and after work-up this recycling process was repeated up to three times.

E. Effect of the Organic Solvents

Seven different organic solvents were added to each Eppendorf vial. The organic solvents used were: dimethyl sulfoxide (DMSO), acetonitrile (MeCN), 1,4-dioxane, tetrahydrofuran (THF), methyl tert-butyl ether (MTBE), toluene, and hexane.

15 mg of *E. coli*/RasADH was added into an Eppendorf vial and dissolved in 240 µL of phosphate buffer 100 mM pH 7.5 and a solution containing 60 µL of phosphate buffer 100 mM pH 7.5 and $NADP^+$ (10 mM). Then, 300 µL of the organic solvent was added to the mixture as well as propiophenone (3 µL) and propan-2-ol (30 µL). The reaction was left in the shaker (250 rpm) for 24h at 30 °C. The aqueous phase was extracted by the addition of EtOAc (2 x 500 µL).

F. Standard Reaction with Oxidoreductases and Glycerol for RasADH and SyADH Reactions

15 mg of *E. coli*/RasADH and *E. coli*/SyADH was added into separate Eppendorf vials and dissolved in 280 µL of phosphate buffer 100 mM pH 7.5 and a solution containing 60 µL of phosphate buffer 100 mM pH 7.5 and $NADP^+$ (10 mM). Then, 260 µL of glycerol was added to the mixture as well as propiophenone (3 µL) and propan-2-ol (30 µL) (Fig. 2). The reaction was left in the shaker (250 rpm) for 24h at 30 °C. The aqueous phase was extracted by the addition of EtOAc (2 x 500 µL).

G. Preparation of the ADH-T, TeSADH, LBADH and ADH-A-Catalyzed Reactions

15 mg of *E. coli*/ADH-T and *E. coli*/TesADH was added into separate Eppendorf vials and dissolved in 280 µL of phosphate buffer 100 mM pH 7.5 and a solution containing 60 µL of phosphate buffer 100 mM pH 7.5 and $NADP^+$ (10 mM). Then, 260 µL of glycerol was added to the mixture as well as acetophenone (3 µL) and propan-2-ol (30 µL). ADH-T and TeSADH are enzymes that reduce acetophenone to 1-phenylethanol (Fig. 3). Also, 15 mg of E. *coli*/LBADH was added to an Eppendorf vial and dissolved in 280 µL phosphate buffer 100 mM pH 7.5 and a solution containing 60 µL phosphate buffer 100 mM pH 7.5 and $NADP^+$ (10 mM). Then, 260 µL of glycerol was added to the mixture as well as acetophenone (3 µL), propan-2-ol (30 µL) and 60 µL of $MgCl_2$ solution (10 mM) prepared in phosphate buffer 100 mM pH 7.5.

15 mg of *E. coli*/ADH-A was added into an Eppendorf vial and dissolved in 280 µL of phosphate buffer 100 mM pH 7.5 and a solution containing 60 µL phosphate buffer 100 mM pH 7.5 and NAD^+ (10 mM). Then, 260 µL of glycerol was added to the mixture as well as acetophenone (3 µL) and propan-2-ol (30 µL). All these reactions were left in the shaker (250 rpm) for 24h at 30 °C. The aqueous phases were extracted by the addition of EtOAc (2 x 500 µL).

H. Preparation of the Blank Reactions

Two blank reactions containing only the lyophilized *E. coli* cells, without the overexpression of any recombinant ADH were performed as follows: 15 mg of *E. coli*, 540 µL of phosphate buffer 100 mM pH 7.5 and a solution containing 60 µL of phosphate buffer 100 mM pH 7.5, NAD^+ (5 mM) and $NADP^+$ (5 mM). Then, acetophenone (3 µL) and propan-2-ol (30 µL), were added. The reaction was left in the shaker (250 rpm) for 24h at 30 °C. The aqueous phase was extracted by the addition of EtOAc (2 x 500 µL).

The other reaction was exactly the same except the substrate, propiophenone (3 µL), instead of acetophenone.

I. I.The Effect of NADP on the Bioreduction Activity of RasADH

To test the effect of NADP on the biocatalysis activity of ADH from *Ralstonia* sp., three reactions were prepared. The first buffer reaction was composed of: 15 mg of *E. coli*/RasADH, 600 µL phosphate buffer 100 mM pH 7.5 and a solution containing 60 µL phosphate buffer 100 mM pH 7.5 and NADP+ (10 mM). Propiophenone (3 µL) and propan-2-ol (30 µL) were also added to the mixture.

The second reaction contained the same components as in the buffer reaction but lacked NADP, and 300 µL of phosphate buffer were added to the mixture in addition to 480 µL of DES (1:2 ChCl/ethylene glycol).

The third reaction composed of the same components as in the second reaction but differed by the addition of 480 µL glycerol instead of the DES solution. The following reactions were prepared to test the effect of recycling the reactions without a cofactor. After extracting the aqueous phase with ethyl acetate, propiophenone (3 µL) and propan-2-ol (30 µL) were added to the organic phases of the three reactions. 14 µL of DES solution was added to the second reaction, and 40 µL *of glycerol was added to the third reaction. The reactions were left in the shaker (250 rpm) for 24h at 30 °C and this recycling was repeated three times.*

J. The Effect of NAD on the Bioreduction Activity of ADHA and ADHT

The ADHs used were from *E. coli* strains that overexpressed the ADH from *Rhodococcus ruber (*ADHA*)* and from *Thermoanaerobiuum* sp. (ADHT) [6]. For the ADHA enzyme, five reactions were prepared, the first reaction contained the buffer (600 µL*)* and the second one contained DES at 50% (300 µL*)*, the third reaction contained des at 80% (480 µL*)*, the fourth contained glycerol at 50% and the fifth contained glycerol at 80%. The same five reactions were prepared for the ADHT enzyme. Each reaction contained 15 mg of the recombinant cells that overexpressed ADHA or ADHT. Propiophenone (3 µL) and propan-2-ol (30 µL) were also added to the reactions.

For the recycling; propiophenone (3 µL) and propan-2-ol (30 µL) were added to the organic phases of the reactions. 14 µL of DES solution was added to the reactions that contained DES, and 40 µL of glycerol was added to the reactions containing glycerol. The reactions were left in the shaker (250 rpm) for 24 h at 30 °C and this recycling was repeated three times. At the end, conversion and enantioselectivity were measured via gas chromatography.

III. Results

This study revealed that standard reactions with the different types of DES at 50% (v/v) reduced better the substrates to the corresponding alcohols, compared to the reactions of DES at 80% (v/v) with RasADH (Table I). However, the reactions with DES at 80% showed a good enantioselectivity of the (*S*)-1-phenylpropanol obtained. These results suggest that when increasing the amount of DES, this medium increases the enantioselectivity of the enzyme but reduces slightly its ability to convert the substrate to the alcohol. It was also observed that the DES reaction that contained urea afforded the worse results in terms of conversion.

When comparing the conversion and the enantioselectivity results between the first recycling and the second and third ones, we noticed that the second one gave worse conversions although slightly better enantioselectivity values.

The best reduction reaction rates were observed in the original reactions (Table I). However, it was noticed that the more the reactions were recycled, the more the conversion decreased. This might be due to the fact that the ADH gets inactivated within the time. On the other hand, the enantiomeric selectivity of the *S* alcohol increased with the number of recycling (entries 6-20, Table I).

Based on the results that were obtained in Table I DES 2 and glycerol can be considered as the best solvents. These two solvents gave a high yield of the desired alcohol, and also a high enantioselectivity of the *S*-alcohol. These findings confirmed the previous work done on this respect, and that glycerol has a strong stabilizing effect on the ADH from *E. coli*/RasADH [5].

When testing the effect of the seven organic solvents on the ADH stability, the best conversion to the alcohol was observed with DMSO at 50% (v/v) and hexane at 80% (v/v). MTBE at 50% (v/v) and toluene at 80% (v/v) gave the best selectivities in the presence of RasADH (Table II).

TABLE I
EFFECT OF GLYCEROL AND DES (80% V/V) ON *E. COLI*/RASADH AT 30 °C

Entry	Medium	Recycling	Conversion (%)	*ee* (%)
1	Buffer	-	94	2
2	Glycerol	-	90	4
3	DES 1	-	91	78
4	DES 2	-	88	66
5	DES 3	-	43	87
6	Buffer	1	85	47
7	Glycerol	1	85	42
8	DES 1	1	48	76
9	DES 2	1	74	77
10	DES 3	1	15	84
11	Buffer	2	60	68
12	Glycerol	2	76	68
13	DES 1	2	18	No data
14	DES 2	2	62	87
15	DES 3	2	9	77
16	Buffer	3	27	90
17	Glycerol	3	74	90
18	DES 1	3	14	No data
19	DES 2	3	58	97
20	DES 3	3	4	No data

DES 1: choline chloride:ethylenglycol (1:2); DES 2: choline chloride:glycerol (1:2); DES 3: choline chloride:urea (1:2)

It was also observed that high temperatures slightly decreased the ability of *E. coli*/RasADH to convert the substrates to the corresponding alcohols and also decreased the ability of the enzyme to give the specific enantiomer (Table III). After the addition of glycerol to the different ADHs, it was

observed that glycerol (50% v/v) enhanced the ability of the enzyme to convert the substrate to the alcohol, but did not afford high enantiomeric excess (Table IV).

TABLE II
EFFECT OF ORGANIC SOLVENTS AT 50% AND 80% (V/V) ON *E. COLI*/RASADH AT 30 °C

Entry	Organic solvent (% v/v)	Conversion (%)	*ee* (%)
1	DMSO (50)	88	72
2	MeCN (50)	3	No data
3	1,4-dioxane (50)	78	72
4	THF (50)	2	No data
5	MTBE (50)	81	84
6	Toluene (50)	66	74
7	Hexane (50)	80	51
8	DMSO (80)	1	25
10	1,4-dioxane (80)	71	38
12	MTBE (80)	79	20
13	Toluene (80)	63	94
14	Hexane (80)	86	64

TABLE III
EFFECT OF TEMPERATURE ON *E. COLI*/RASADH IN THE PRESENCE OF DES

Entry	Temperature (°C)	Medium	Conversion (%)	*ee* (%)
1	45	Buffer	94	4
2	45	DES 1	90	80
3	45	DES 2	89	57
4	45	DES 3	32	86
5	45	Glycerol	92	88
6	60	Buffer	66	35
7	60	DES 1	44	67
8	60	DES 2	44	41
9	60	Glycerol	90	84

TABLE IV
GLYCEROL EFFECT ON DIFFERENT ADHS

Entry	Enzyme	Conversion (%)	*ee* (%)
1	ADH-A in buffer	80	>99
2	ADH-A in glycerol	80	42
3	ADH-T in buffer	80	>99
4	ADH-T in glycerol	79	42
5	LBADH in buffer	82	88
6	LBADH in glycerol	79	92
7	TeSADH in buffer	68	70
8	TeSADH in glycerol	75	90
9	SyADH in buffer	75	24
10	SyADH in glicerol	85	>99

When testing the effect of the recycling on the different ADHs, the conversion of the substrate to the alcohol decreased with time, and the enantioselectivity of the enzyme was maintained or slightly diminished (Table V). However, when we increased the temperature, and added glycerol (50% v/v), the different enzymes generally gave good conversions and good *ee* (Table VI). It must be mentioned that the blank reactions with the wild-type *E. coli* without any ADH overexpressed did not convert the ketones to the corresponding alcohols, showing that the activities and selectivities obtained came from the overexpressed ADHs.

TABLE V
RECYCLING EFFECT ON DIFFERENT ADHS

Entry	Enzyme	Recycling	Conversion (%)	*ee* (%)
1	ADH-A	1	75	>99
2	ADH-T	1	81	>99
3	LBADH	1	79	>99
4	TeSADH	1	42	86
5	SyADH	1	42	99
6	ADH-A	2	77	99
7	ADH-T	2	78	99
8	LBADH	2	79	58
9	TeSADH	2	20	56
10	SyADH	2	18	62
11	ADH-A	3	78	90
12	ADH-T	3	79	98
13	LBADH	3	78	94
14	TeSADH	3	10	74
15	SyADH	3	6	83

TABLE VI
TEMPERATURE EFFECT ON DIFFERENT ADHS IN THE PRESENCE OF GLYCEROL (50% V/V)

Entry	Enzyme	Temperature (°C)	Conversion (%)	*ee* (%)
1	ADH-A	45	81	98
2	ADH-T	45	82	96
3	LBADH	45	83	78
4	TeSADH	45	78	84
5	SyADH	45	82	54
6	ADH-A	60	87	98
7	ADH-T	60	85	>99
8	LBADH	60	89	80
9	TeSADH	60	75	76
10	SyADH	60	68	99

TABLE VII
NADP EFFECT ON THE BIOREDUCTION ACTIVITY OF RASADH

Sample	1st recycling		2nd recycling		3rd recycling	
	C%	*ee* (%)	C%	*ee* (%)	C%	*ee* (%)
(*E. coli*/RasADH) in buffer	36	28	14	30	5	32
(*E. coli*/RasADH) in DES 80%	63	64	45	74	39	86
(*E. coli*/RasADH) in glycerol 80%	60	46	37	66	44	84

TABLE VIII
NAD EFFECT ON THE BIOREDUCTION ACTIVITY OF ADHA AND ADHT

Sample	1st recycling		2nd recycling		3rd recycling	
	C%	*ee* (%)	C%	*ee* (%)	C%	*ee* (%)
ADHA buffer	35	>99	10	>99	4	>99
ADHA DES 50 %	76	>99	61	>99	62	>99
ADHA DES 80 %	71	>99	51	>99	48	>99
ADHA glycerol 50 %	78	>99	59	>99	55	>99
ADHA glycerol 80%	78	>99	40	>99	32	>99
ADHT buffer	31	>99	16	>99	8	>99
ADHT DES 50 %	38	>99	13	>99	10	>99
ADHT DES 80 %	41	>99	22	>99	19	>99
ADHT glycerol 80%	79	>99	76	>99	75	>99
ADHT glycerol 80%	77	>99	80	>99	85	>99

Compared to the original reactions, it was found that as we

recycled the reactions without the NADP coenzyme, the enzyme's ability to convert the substrate to the corresponding alcohol decreased. But, as the reactions were recycled, this increased the enantioselectivity of the enzyme (Table VII). These findings were also observed with the other enzymes (ADHA and ADHT), when NAD was not added (Table VIII).

The enantiomeric excess (*ee*) was calculated by:

$$ee\ (\%) = \frac{(S-R)\times 100}{100}$$

where, S: the area of the more abundant enantiomer (*S*). R: the area of the less abundant enantiomer (*R*).

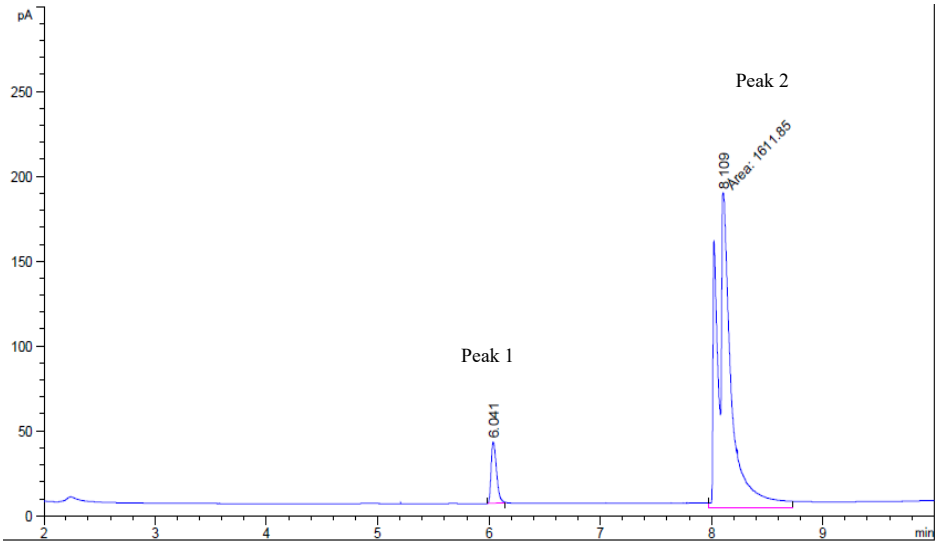

Fig. 2 Gas chromatography of 1-phenylpropanol (Rt= 8.1 min) from the reduction of propiophenone (Rt= 6.0 min) by RasADH. Peak 1: Rt=6, Peak 2: Rt=8.1

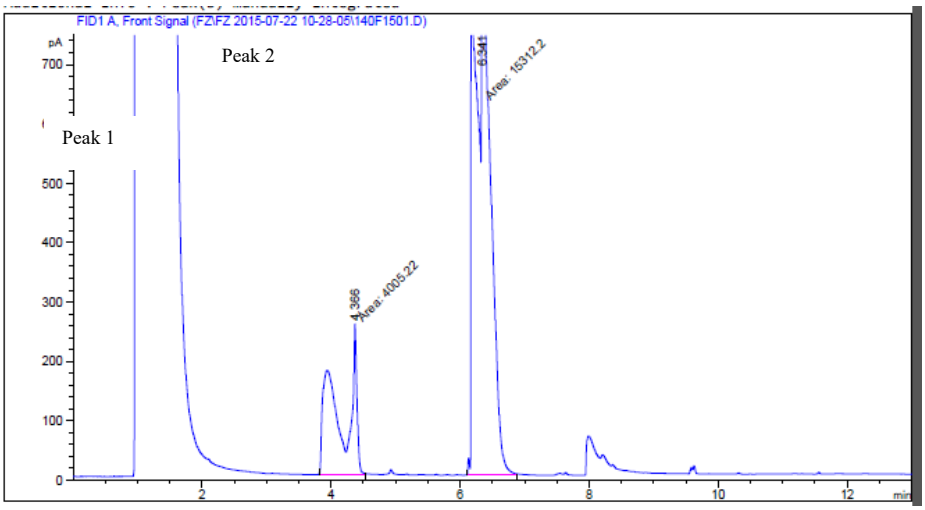

Fig. 3 Gas chromatography of 1-phenylethanol (Rt= 6.3 min), after the reduction of acetophenone (Rt= 4.4 min) by ADH-T from *Thermoanaerobium* sp. Peak 1: Rt=4.3, Peak 2: Rt=6.3

IV. Conclusion

In conclusion, the reactions containing the ADHs in the presence of DES could be recycled in a more efficient manner, affording better conversions and maintaining or improving the enantiomeric excess obtained with reactions in plain buffer. These findings confirmed the work that was done to test the efficiency of DES solvents with the ADHs enzymes [6]. In addition to that, glycerol maintained also the ADHs stable in most of the reactions. For this reason, a good conversion and enantiomeric excess into the (*S*)-alcohols were obtained. It was also observed that temperature disabled the stabilizing effect of glycerol and decreased the ability of the enzyme to give the *S* enantiomer of the alcohol, although again at a lower extent as compared to the plain buffer system.

It was also observed that, when temperature increased, the ability of RasADH to convert propiophenone to 1-

phenylpropanol decreased, also affecting the selectivity of the process. However, a temperature increase has an opposite effect on ADHs from other sources.

One of the objectives of this study, which tackled different ADHs, was to obtain both high conversion and enantiomeric excess while maintaining the enzymes active over higher reaction times and cycles, and this was observed when glycerol or DES 2 was added to the reactions [7]. In addition to that, ADH-T from *Thermoanaerobium* sp. gave the best conversion and enantiomeric excess results in the presence of glycerol at high temperatures.

Based on the results, (*E. coli*/RasADH) has its own NADP, because it was able to convert the substrate in the absence of an additional NADP. Also, the reactions that contained DES as a solvent gave the best conversion and enantioselectivity results, and this suggests the importance of these solvents in enzymatic reactions.

It was also observed that as we recycled the reactions (Table VII), the enantioselectivity got better, and this might be due to the residual substrate that was left in the original reaction container.

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