Enhancement of the Thermostability of *Candida cylindracea* Lipase by Medium Engineering*

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The possibility to increase the thermostability of the enzyme *Candida cylindracea* lipase was studied using the medium engineering method. The purpose was to shift the property of the enzyme obtained from mesophilic organism towards thermophilic character and hence widen its application possibilities. Applying the heptane solvent as reaction medium, the relation between the water content of the reaction mixture and the enzyme activity was investigated in the temperature range of 30 °C and 50 °C. It was found that decreasing the water content of the reaction mixture, the activity of the enzyme was reduced, while its thermostability increased. The slower deactivation of the enzyme was explained by the reduced amount of water present, necessary for the denaturation reactions.

In the last decades a great diversity of microorganisms that can live under extreme conditions has been discovered. These microorganisms are called extremophiles [1—3]. They are adapted to living at very high or low temperatures, pH values, at high salinity or at high pressure. They tolerate not only this extreme environment, but they require it for their growth. Most of them belong to the strain *Archaea*, and they can be classified in the following way:

Thermophiles: grow at 50—80 °C
Hyperthermophiles: grow over 80 °C
Psichrophiles: grow under 20 °C
Acidophiles: grow at pH 0—1
Alcaliphiles: grow at pH 10—11
Barophiles: grow at high pressure
Halophiles: grow at high salinity

These groups and their enzymes provide numerous potentials for biotechnological utilization, especially in case of thermophiles. The latter are advantageous because their enzymes can be used to catalyze reactions at elevated temperature. The increase of temperature is accompanied by an increase in reaction rate, diffusion coefficient, bioavailability and solubility of some organic substrates [4—6].

It is proved that the properties of the mesophilic organisms and their enzymes can be modified similar to extremophile's ones. There are two possible methods for modification: protein engineering (tailoring enzyme function to the reaction) and medium or solvent engineering (tailoring solvent properties to achieve desired reactivity and selectivity) [7].

It is known from the literature that application of organic solvents – instead of water – has several advantages [8—10]: increased solubility of nonpolar solvents; shifting thermodynamic equilibrium to favour synthesis over hydrolysis; suppression of side reactions caused by water; alteration of substrate and enantioselectivity; elimination of microbial contamination; enzymes can be recovered by simple filtration.

Although organic solvents were used as reaction media in these studies, some water was needed to the enzyme to be active. The optimal amount of water depends on the hydrophobicity of the applied solvent [11]. It is well known, on the other hand, that numerous reactions, leading to the denaturation of enzyme, need the presence of some water. Altogether only one paper has been dealing with the effect of water content on the thermostability of the enzyme so far. It was found that the enzyme studied kept its activity at 100 °C for some hours [12].

The aim of our experiments was to increase thermostability of a mesophilic enzyme, *Candida cylindracea* lipase (E.C. 3.1.1.3.) by organic solvents and very small amounts of water in the reaction mixture. Thermostability of this lipase was studied earlier only at 0.4 % water content, which was found optimal from the reaction rate point of view [11]. According to the results of those experiments it could be stated that

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the enzyme showed the highest activity at 30—40 °C and it was quickly denatured above 50 °C.

The test reaction chosen was an enantioselective esterification catalyzed by *Candida cylindracea* lipase [13]. Racemic mixture of 2-chloropropanoic acid and butan-1-ol was converted into (*R*)-2-chloropropanoic acid butyl ester by the enzyme. (*S*)-2-Chloropropanoic acid remained in the reaction mixture without any change. As the reaction progresses, more and more water is formed, which can shift the state of the equilibrium towards hydrolysis. Several methods are known for water removal produced during the reaction [14], but application of these processes had not made it possible to study correctly the relations between water and enzyme.

EXPERIMENTAL

Candida cylindracea lipase (nominal activity: 943 U/mg enzyme) was from Sigma (St. Louis, MI, USA). Butan-1-ol, hexane, heptane, and Karl-Fischer solution were from Reanal (Budapest, Hungary), 2-chloropropanoic acid was from Merck (Darmstadt, Germany).

The required reaction temperature and the homogenization of reaction mixture were provided in a New Brunswick G24 shaking incubator. Experiments were carried out at 150 min^{-1} in the temperature range of 30—50 °C. 3 trials were performed in 100 cm^3 flasks filled with 25 cm^3 reaction mixture. Flasks containing reactants with a mole ratio 6 : 1 for butan-1-ol (0.06 mol) and 2-chloropropanoic acid (0.01 mol) were placed into the shaking incubator preheated for the required temperature for some minutes. Then 0.05 g of enzyme was added to the mixture and flasks were shaken with 150 min^{-1}. Samples were taken after 1 h, 2 h, 3 h, 4 h, and 5 h. Before taking samples the shaking incubator was stopped and the enzyme precipitated at the bottom of the flask in some seconds.

Initial water content of the reaction mixture was determined by a Mettler DL35 automatic Karl-Fischer titrator.

The esterification reaction was followed by an HP 5890A type GC using a 25-m FS-LIPODEX E chiral capillary column (0.25 mm i.d., thickness of the film: 0.2 μm). Analysis parameters were the following: volumetric flows of carrier and auxiliary nitrogen were 2.2 $\mathrm{cm}^3\ \mathrm{min}^{-1}$ and 27.8 $\mathrm{cm}^3\ \mathrm{min}^{-1}$, respectively. Hydrogen flow was set to 25 $\mathrm{cm}^3\ \mathrm{min}^{-1}$, air flow was 230 $\mathrm{cm}^3\ \mathrm{min}^{-1}$. The blow off rate based on previous data was 1 : 45. Temperature of both injector and detector was 250 °C. The computer kept column space temperature at 100 °C for 5 min, then heated it up to 180 °C with the rate of 10 °C min^{-1} and finally kept it at this value for 10 min. From each sample 0.5 mm^3 was injected onto the gas chromatography column and the amount of (*R*)-2-chloropropanoic acid butyl ester produced in the reaction was measured.

At first optimal parameters of the analysis were set and calibration curve was determined for evalu-

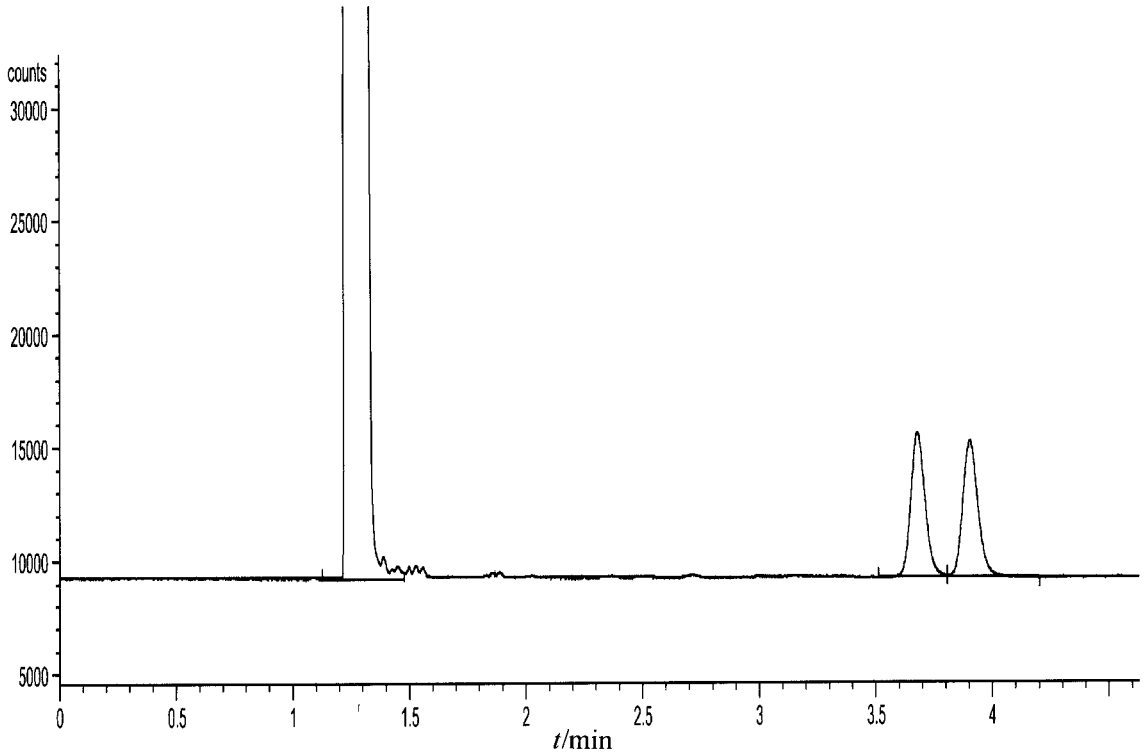

Fig. 1. Chromatogram of the racemic 2-chloropropanoic acid butyl ester in heptane. Analysis conditions see in the text.

ating chromatograms. For this task calibration solutions were prepared from racemic 2-chloropropionic acid butyl ester standard, the concentrations of which correspond to 20 %, 40 %, 60 % conversions. Peaks specific to the observed ester isomers appeared at 3.7 min and 3.9 min retention time, respectively. Fig. 1 shows a typical chromatogram of the racemic mixture in heptane solvent.

RESULTS AND DISCUSSION

Since the aim of this work was to carry out the test reaction at elevated temperatures, hexane, the commonly used solvent was not appropriate because of its low boiling point. The selection of a proper solvent was the first step in the experimental work. A solvent had to be found that ensures appropriate enzyme activity, dissolves ester produced in the reaction and due to its boiling point it is allowed to use elevated temperatures. Heptane (boiling point: 98.42 °C) was found to be the most appropriate solvent. In our further experiments this solvent was used as organic medium.

Experiments were carried out according to the description above at 30 °C, 40 °C, and 50 °C. Enzyme activity was investigated at initial water contents of 0.4 %, 0.2 %, 0.1 %, and 0.05 % at each temperature.

Fig. 2 shows that a maximal conversion at 30 °C was achieved applying 0.2 % initial water content. This value is different from those commonly given by other authors [15] which are about 0.4 % of initial water. The discrepancy can be explained that in these works either the reaction was studied up to only 10 % conversion, or the water produced was removed continuously. In our experiments, the water produced during the reaction elevated the water content of the reaction mixture and in case of 0.4 % initial water content, it was found high enough to influence unfavourably the reaction rate.

Fig. 3 shows that the initial phases (up to the first hour) of the progress curves obtained at 40 °C and four different initial water contents were almost identical. The conversions in the cases of 0.05 %, 0.1 %, and 0.2 % initial water content increased further, while at 0.4 % initial water content the conversion remained at a constant value of 24 %.

In Fig. 4, the conversion data at 50 °C are presented. It can be observed that the conversion increases with the decreasing of water content. It can be seen that using 0.4 % water content the conversion – similarly to the data measured at 40 °C – attains a constant level. From this phenomenon it can be concluded that the enzyme has been quickly deactivated in the presence of high water content. In the experiments using the lowest 0.05 % initial water content, the conversions reached were the highest, almost 50 %. It is interesting to study the progress of conversion *vs.* time curves as well, since the reaction starts slowly, probably due to the fact that there is not enough water present for the optimal action of the enzyme. As a result of the water produced during the esterification, the reaction is getting faster later on. To learn

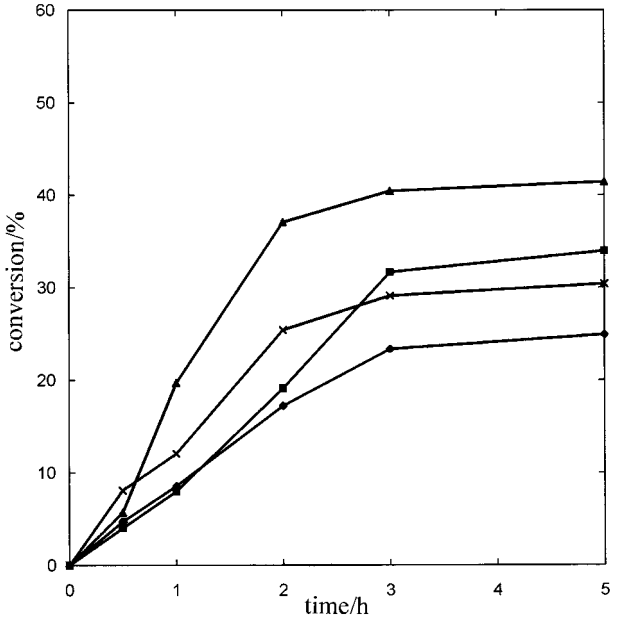

Fig. 2. Conversion of (*R*)-2-chloropropanoic acid in heptane at 30 °C. Experimental data: 2-chloropropanoic acid (0.01 mol) and butan-1-ol (0.06 mol) in 25 cm^3 solvent, 0.05 g *Candida cylindracea* lipase, water content: ♦ 0.05 %, ■ 0.1 %, ▲ 0.2 %, × 0.4 %, other parameters see in the text.

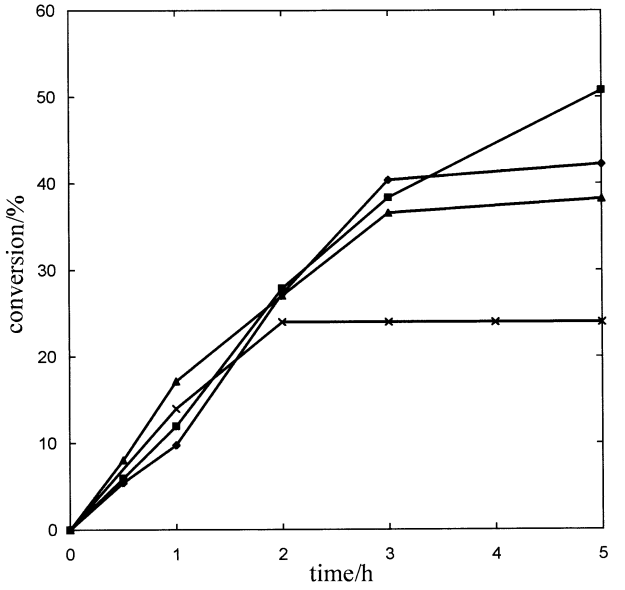

Fig. 3. Conversion of (*R*)-2-chloropropanoic acid in heptane at 40 °C. Experimental data: 2-chloropropanoic acid (0.01 mol) and butan-1-ol (0.06 mol) in 25 cm^3 solvent, 0.05 g *Candida cylindracea* lipase, water content: ♦ 0.05 %, ■ 0.1 %, ▲ 0.2 %, × 0.4 %, other parameters see in the text.

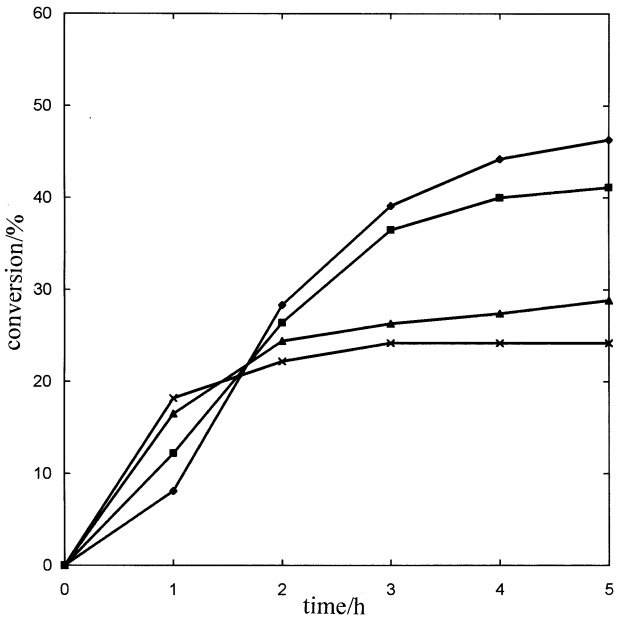

Fig. 4. Conversion of (*R*)-2-chloropropanoic acid in heptane at 50 °C. Experimental data: 2-chloropropanoic acid (0.01 mol) and butan-1-ol (0.06 mol) in 25 cm^3 solvent, 0.05 g *Candida cylindracea* lipase, water content: ♦ 0.05 %, ■ 0.1 %, ▲ 0.2 %, × 0.4 %, other parameters see in the text.

the phenomenon thoroughly, further, detailed investigations are needed at higher temperature and with lower water content.

The phenomena above can be explained by the fact that increasing the temperature the enzyme molecule is reversibly enlarging, then one or more of the following reactions take place, which may cause the irreversible change of the enzyme structure:

– splitting of the S—S bonds by β-elimination;

– deamidation of asparagine and glutamine residues;

– hydrolysis of peptide bonds in asparagine residues.

Water is needed for these reactions, therefore it can be assumed that in low-water-content media these reactions take place considerably slower. Therefore it is an important task to determine the optimal water content and to keep its level at a constant value by the one of the well-known methods (*i.e.* adsorption, pervaporation, azeotropic distillation).

CONCLUSION

The first experiments in application of medium engineering for enhancing the thermostability of enzymes have proved that varying the initial water content of the reaction mixture the activity of *Candida cylindracea* lipase could be influenced. In the course of the experiments in heptane solvent, the enzyme thermostability has increased with reducing the water content, which is shown very well by the results obtained in the experiments at 50 °C. Further experiments are planned to be carried out at higher temperature and in other solvents with various hydrophobicities. In the latter case the same amount of water is distributed between the solvent and the enzyme in different ways, because of the distinct hydrophobicities of the solvents used. Hence this phenomenon may serve as a further evidence of the role of water in denaturation.

The aim of our further experiments is to substitute the water necessary for the enzyme with water-mimicking additives capable to form hydrogen bonds. Thus the amount of water present in the media can be decreased to a minimal value and reactions causing denaturation of the enzyme can be avoided. In this way the application range of the lipase can be noticeably widened, especially for the environmental processes.

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REFERENCES

1. Van der Ost, J. and De Vos, W. M., *Trends Biotechnol. 14*, 415 (1996).
2. Madigan, M. T. and Oren, A., *Curr. Opin. Microbiol. 2*, 265 (1999).
3. Nawani, N., Dosanjh, N. S., and Kaur, J., *Biotechnol. Lett. 10*, 997 (1998).
4. Sellek, G. A. and Chaudhuri, J. B., *Enzyme Microb. Technol. 25*, 471 (1999).
5. Scandurra, R., Consalvi, V., Chiaraluce, R., Politi, L., and Engel, P. C., *Biochimie 80*, 933 (1998).
6. Niehaus, F., Bertoldo, C., Kähler, M., and Antranikian, G., *Appl. Microbiol. Biotechnol. 6*, 711 (1999).
7. Dordick, J. S., *Biotechnol. Prog. 8*, 259 (1992).
8. Klibanov, A. M., *Tibtech 15*, 97 (1997).
9. Rich, J. O. and Dordick, J. S., *J. Am. Chem. Soc. 119*, 3245 (1997).
10. Wehtje, E. and Adlercreutz, P., *Biotechnol. Bioeng. 55*, 798 (1997).
11. Gubicza, L. and Szakács-Schmidt, A., *Biocatalysis 9*, 131 (1994).
12. Zaks, A. and Klibanov, A. M., *Proc. Natl. Acad. Sci. U.S.A. 82*, 3192 (1985).
13. Ulbert, O., Szarka, Á., Halasi, Sz., Somogyi, B., Bélafi-Bakó, K., and Gubicza, L., *Biotechnol. Tech. 13*, 299 (1999).
14. Gubicza, L. and Szakács-Schmidt, A., *Biotechnol. Tech. 9*, 687 (1995).
15. Kirchner, G., Scollar, M. P., and Klibanov, A. M., *J. Am. Chem. Soc. 107*, 7072 (1985).