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RESEARCH ARTICLE

# Lipase-catalyzed enantioselective esterification of flurbiprofen with n-butanol

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**The influences of water activity and solvent hydrophobicity on the kinetics of the lipase-catalyzed enantioselective esterification of flurbiprofen with n-butanol were investigated. The solvent effect was not similar for lipases from *Candida rugosa* (Crl), *Mucor javanicus* (Mjl), and porcine pancreas (Ppl). The lipase-catalyzed reaction rates in different solvents across a wide range of water activities revealed that the Ppl-catalyzed reaction exhibited no enantioselectivity and no substantial water activity or solvent dependence. The Mjl-catalyzed reaction proceeded faster, preferring the R-enantiomer reaction over that of its antipode, but had little solvent or water activity dependence. The Crl-catalyzed reactions in n-heptane and n-nonane had similar water activity dependence, but the reaction was considerably faster and more enantioselective (preferring the S-enantiomer reaction) in isooctane, a solvent whose hydrophobicity is intermediate between that of the other two alkanes. Substrate enantiomeric excess, for the Crl-catalyzed reaction at 96 hours and at a water activity of 0.65, in n-heptane, isooctane, and n-nonane was 40.9, 93.0, and 50.0%, respectively. Since the three solvents possess similar physical properties, the explanation for this anomalous behavior might be the effect of the solvent structural characteristics on Crl, since isooctane is the only branched alkane.**

It has been well established that enantiomers of non-steroidal anti-inflammatory drugs of the aryl propionic class exhibit diverse pharmacological and toxicological properties. The therapeutic action of racemic mixtures of these drugs is mainly due to the (S)-isomers (Shen, 1972). It would be advantageous and safer to administer only the enantiomer with the desirable properties, giving rise to the need for simple, inexpensive methods to obtain the individual enantiomers in high chemical and optical yields.

Kinetic resolution of racemic mixtures by enzymes in organic media has been used successfully as a method for enantiomeric synthesis (Klibanov, 1990). Members of the lipase family have been found to be particularly suitable for such applications, and lipases from *Candida cylindracea, Candida antarctica,* porcine pancreas, and other sources have been used to resolve the enantiomers of naproxen (Tsai and Wei, 1994), ibuprofen (Mustranta, 1992; Tsai et al. 1997), and suprofen (Mertoli et al. 1996).

The S-enantiomer of flurbiprofen (2-fluoro-α-methyl-[1,1'-biphenyl]-4-acetic acid) possesses most of its anti-inflammatory action, but the presence of the R-enantiomer is reported to greatly enhance its gastrointestinal toxicity (Wechter et al. 1993). A later study reported that R-flurbiprofen was less potent as an analgesic than S-flurbiprofen, but it caused little toxicity in comparison to its antipode (Lotsch et al. 1995). This makes the resolution of the enantiomers of this drug particularly desirable. The lipase-catalyzed enantioselective esterification of racemic flurbiprofen has been reported (Morrone et al. 1995). The

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factors known to profoundly affect the reaction rate and enantioselectivity are solvent hydrophobicity (Laane et al. 1987; Parida and Dordick, 1993), water content or water activity (Valivety et al. 1992), and the type of lipase (Mustranta, 1992). The present study focuses on determining whether the enantioselective esterification reaction of flurbiprofen with n-butanol, as shown for *Candida rugosa* lipase in Figure 1, can be improved by using hydrophobic solvents, varying the water activity, and using lipases from different sources.

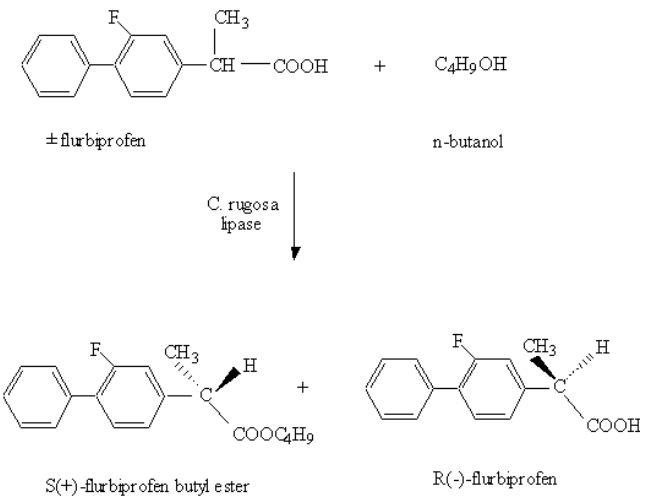


**Figure 1. Reaction scheme for the enantioselective esterification of flurbiprofen with n-butanol.**

## Materials and Methods

### Materials

All lipases used in this study were commercial preparations and were used as received. *Candida rugosa* lipase (Crl) and porcine pancreas lipase (Ppl) were purchased from Sigma Chemical Co. (St. Louis, MO), and *Mucor javanicus* lipase (Mjl) was obtained from Fluka Chemical Co. (Ronkonkoma, NY). Caffeine, which was used as the internal standard for HPLC analysis, was purchased from Eastman Kodak Co. (Rochester, NY).

All solvents chosen had high log P (logarithm of n-octanol-water partition coefficient) values and the influence of increasing hydrophobicity was evaluated. The solvents used were toluene (log P = 2.5), n-heptane (log P = 4.0), isooctane (log P = 4.5), n-nonane (log P = 5.1). All solvents were obtained from Fisher Scientific Co. (Pittsburgh, PA). Water activities in the reaction mixtures were controlled using salt hydrates as described by Halling (Halling, 1992). Water activities in the range 0.18 to 0.85 were controlled using the following salt hydrate pairs: $Na_2HPO_4 \cdot 12H_2O/7H_2O$ ($a_W$ = 0.85), $Na_2HPO_4 \cdot 7H_2O/2H_2O$ (0.65), $SrCl_2 \cdot 6H_2O/2H_2O$ (0.37), and $Na_2HPO_4 \cdot 2H_2O/0H_2O$ (0.18).

### Reaction conditions

The reaction mixture consisted of 300 mg enzyme, 500 mg of each of the appropriate salt hydrates, and 10 ml of a stock solution consisting of 0.6 mM racemic flurbiprofen and 50 μM caffeine dissolved in the solvent under study. The reaction mixture was allowed to equilibrate at the desired water activity by shaking in a constant temperature shaker bath (Blue M Magniwhirl) at 30°C for 24 hours. The mild temperature and solvent medium conditions were unlikely to compromise the stability of the enzymes over the duration of the reaction. Eleven microliters of n-butanol were added to the reaction vessel to obtain a concentration of 12 mM n-butanol and to start the reaction. The reaction mixture was then shaken in the constant temperature shaker bath at 30°C for an additional 24 hours. Twenty five microliter samples were taken at predetermined time intervals. All experiments were conducted at least in quadruplicate, and outliers were removed from a data set using established statistical techniques.

### Analytical methodology

The LC system consisted of an Altex Model 110A pump, a Spectroflow 757 UV detector, a Dionex 4270 integrator and a Rheodyne 7125 injector. Enantiospecific HPLC analysis of flurbiprofen enantiomers in the samples was accomplished using a chiral $\alpha_1$-acid glycoprotein column by modifying a literature method (Geisslinger et al. 1992). The mobile phase consisted of 4% 2-propanol and 4 mM N,N-dimethyloctylamine in 20 mM potassium phosphate buffer adjusted to pH 6.5. The flow rate was 1.1 ml/min and the wavelength of detection was 234 nm. Samples were prepared by evaporation of solvent under vacuum and reconstitution of residue with 100 μL of mobile phase. A representative chromatogram is shown in Figure 2. Disappearance of each of the two enantiomers was calculated using their respective peak areas. The initial rate and the enantiomeric excess at 96 hours were calculated for each set of conditions studied.

### Reaction parameters

The initial rate is the absolute value of the slope of the initial linear portion of the hyperbolic curve obtained by plotting substrate concentration as a function of time, and is reported in units of nmol/(g of enzyme•hr). The initial rate of the faster reacting enantiomer is presented in the Results and Discussion section. The enantiomeric excess of the substrate, $ee_S$, at a given time was calculated using:

$$ee_S = \frac{[B] - [A]}{[A] + [B]}$$

where [A] and [B] are the molar concentrations of the faster and slower reacting enantiomer respectively. The extent of conversion at any given time, c, was calculated using:

$$c = 1 - \frac{[A] + [B]}{[A]_o + [B]_o}$$

where the subscript o denotes initial concentrations.

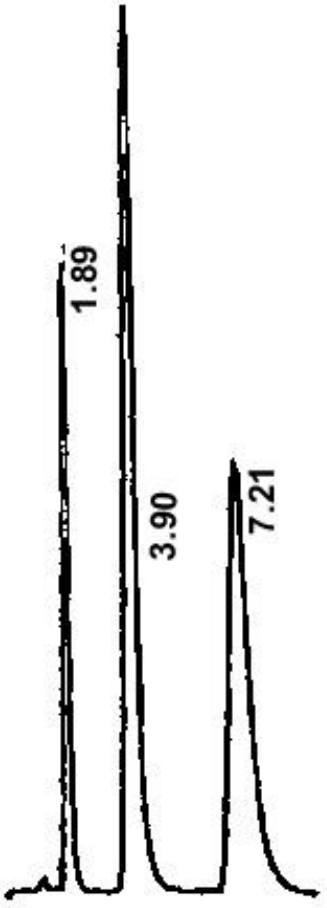


**Figure 2. Representative HPLC chromatogram for flurbiprofen analysis: caffeine (1.89 min), R-flurbiprofen (3.90 min), and S-flurbiprofen (7.21 min).**

## Results and Discussion

### Solvent dependence

It has been established that increasing hydrophobicity exponentially increases the rate of lipase-catalyzed reactions (Laane et al. 1987). The effectiveness of enzyme biocatalysis in organic solvents improves, as a rule, as the log P of the solvent increases, but only up to a limit (Laane et al. 1987). One of the goals of this study was to find the limiting solvent hydrophobicity above which the initial rate for the lipase-catalyzed esterification of flurbiprofen with n-butanol cannot be further enhanced. Preliminary studies showed that the reaction proceeded slowly in toluene (no measurable substrate loss in 96 hours) and faster in isooctane than in n-heptane with *Candida rugosa* lipase. If the higher reaction rate is due to the increased hydrophobicity of the solvent, then it should increase further with n-nonane. However, as can be seen in Figure 3, the *C. rugosa* lipase catalyzed reaction in n-nonane proceeded at about the same rate as in n-heptane. It would seem that a limiting solvent hydrophobicity has been reached, but some factor other than hydrophobicity might be affecting the reaction in isooctane.

Before speculating on what distinguishes isooctane from the other two solvents, it is worth noting if this phenomenon is also observed with the other lipases used in this study. With the other two enzymes, no significant differences in reaction rates were observed in the three solvents, as shown in Figure 4 for *Mucor javanicus* lipase and in Figure 5 for porcine pancreatic lipase. It seems that for both of these lipases, the limiting solvent hydrophobicity has been reached. It would also seem that these lipases were not affected by the nature of the solvent medium in exactly the way *C. rugosa* lipase is.

### Enantioselectivity

The solvent dependence of the enantioselectivity of the enzymes parallels the manner in which their catalytic efficiency depends on solvent hydrophobicity. For *Candida rugosa* lipase, the reaction with the S-enantiomer as substrate proceeds much faster than that of the R-enantiomer for all three solvents, but enantioselectivity is clearly greatest in isooctane (Figure 6). The *Mucor javanicus* lipase favors the R-enantiomer reaction, but shows no solvent effect on enantioselectivity (Figure 7). The porcine pancreas lipase exhibits no significant preference for either enantiomer under any of the conditions studied (data not shown).

The results seem to indicate that a limiting hydrophobicity of the solvent medium exists beyond which increasing hydrophobicity will result in neither improved reaction rate nor improved enantioselectivity. Using solvents more hydrophobic than the ones used in this study might pose practical problems since they tend to be quite viscous. Additionally, if the reaction medium is insufficiently agitated, diffusion problems will lead to slower reactions. The anomalous behavior of *C. rugosa* lipase in isooctane seems to indicate that this enzyme has structural features that make it sensitive to differences in solvent spatial characteristics. Isooctane (2,2,4-trimethylpentane) is the only alkane solvent used in this study that is not a linear alkane.

### Water activity

The influence of water activity on the enzyme catalyzed reaction is somewhat inconsistent. In the case of *M. javanicus* lipase, the effect is erratic but significant (Figure 4). Only in the case of *C. rugosa* lipase-catalyzed reactions in isooctane is it clear that an optimum initial rate exists around a water activity of 0.65 (Figure 3). This behavior is expected since it is generally considered that at lower water

activities the enzyme is inadequately hydrated, and at higher water activities too much water in the medium causes the enzymes to aggregate (Valivety et al 1992). However, after accounting for experimental error, in the case of the *C. rugosa* lipase-catalyzed reactions in the other solvents, and those of the remaining two enzyme preparations, no substantial advantage was gained by maintaining water activity at a specific level in the range studied. The results of this study cannot be directly compared to the prior study on the enzyme-catalyzed esterification of flurbiprofen (Morrone et al. 1995), because the enzyme (the immobilized *Candida antarctica* lipase preparation - Novozym® 435), acyl acceptor (n-propanol), alcohol/acid ratio (three), mass of enzyme (100 mg/ml), and temperature (45°C) were all different. Moreover, a high concentration of substrate (10 mg/ml) was used in that study, much higher than can be achieved in solvents as hydrophobic as some used in the present study. The solvents employed in the

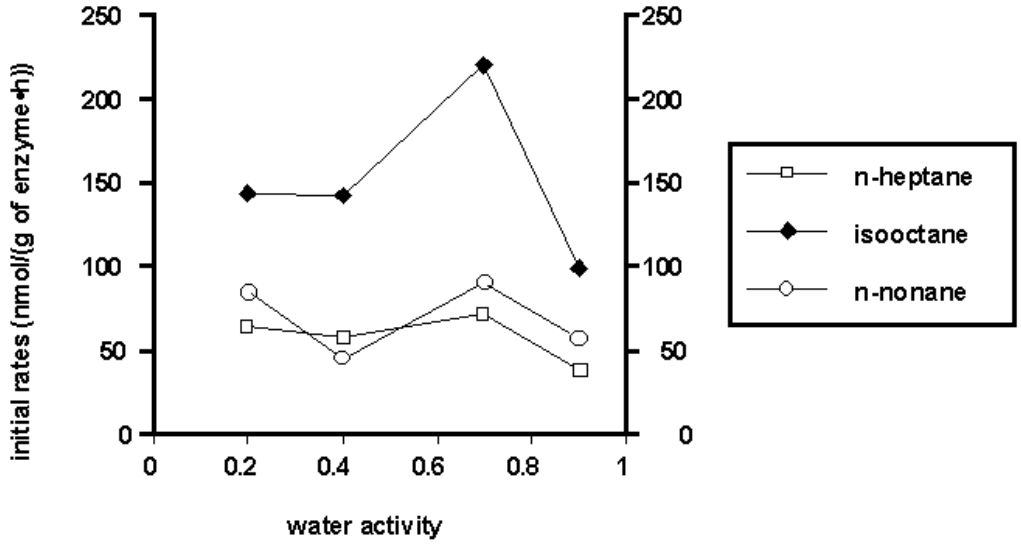


**Figure 3. Initial rates of the *Candida rugosa* lipase-catalyzed reactions.**

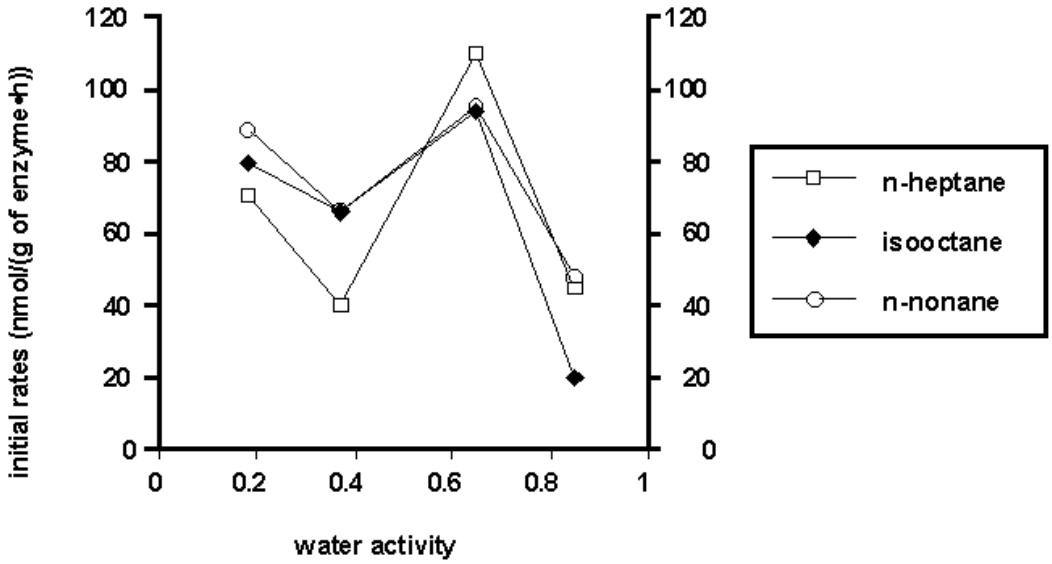


**Figure 4. Initial rates of the *Mucor javanicus* lipase-catalyzed reactions.**

Morrone et al. (1995) study included dioxane (log P = -0.42), acetonitrile (-0.34), tetrahydrofuran (0.49), *tert*-amyl alcohol (0.89), *tert*-butyl methyl ether (1.35), toluene (2.50), and cyclohexane (3.20).

The *C. rugosa* lipase catalyzed reaction in isooctane at a water activity of 0.65 is the fastest (Figure 3), and most enantioselective reaction (Figure 6) of the ones studied.

The reaction is about 79% complete in 72 hours with an enantiomeric excess of 93% of R-flurbiprofen (Figure 8 and Figure 9). It is recommended that the *M. javanicus* lipase catalyzed reaction in n-heptane be used to produce an enantiomeric excess of S-flurbiprofen.

In conclusion, the results of this study suggest that the enantioselective esterification of flurbiprofen catalyzed by lipases is significantly influenced by the nature of the solvent, the source of the lipase, and the activity of water in the reaction medium. In order to optimize the reaction, careful consideration would have to be given to these factors.

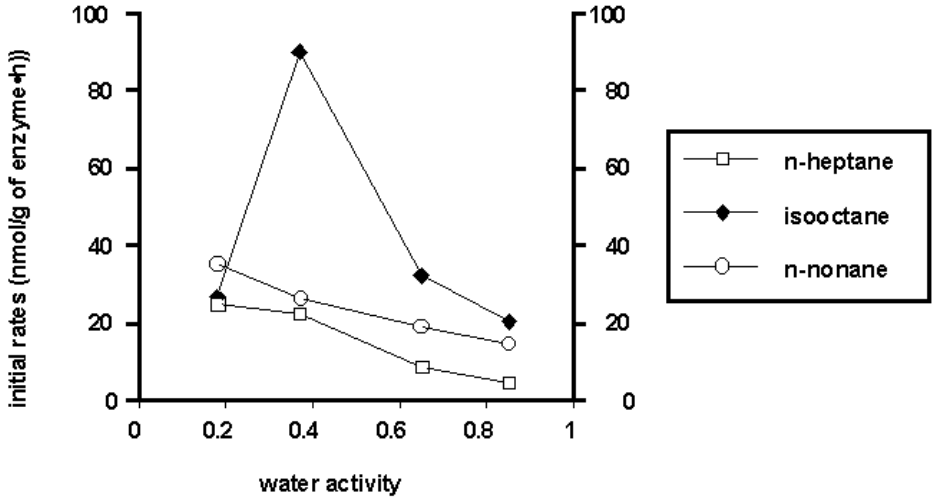


**Figure 5. Initial rates of porcine pancreas lipase-catalyzed reactions.**

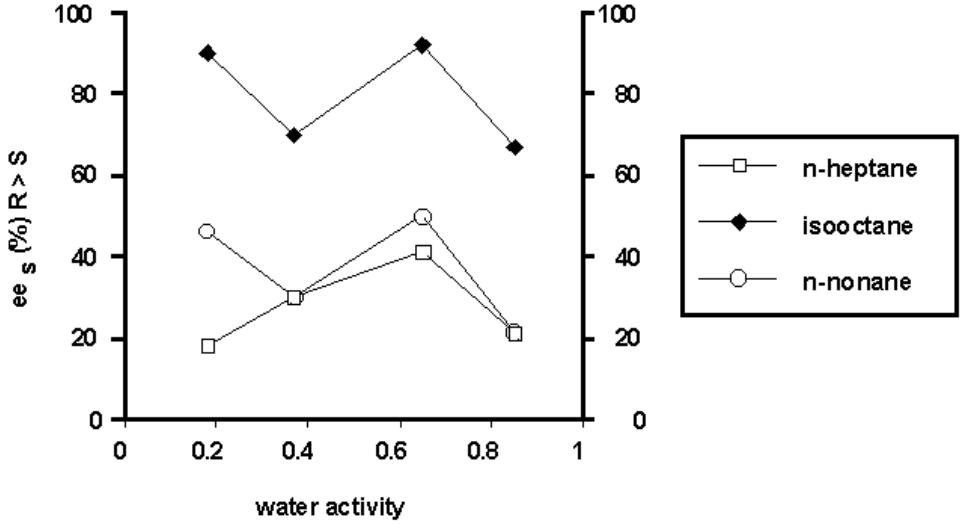


**Figure 6. Enantiomeric excess of the *Candida rugosa* lipase-catalyzed reactions.**

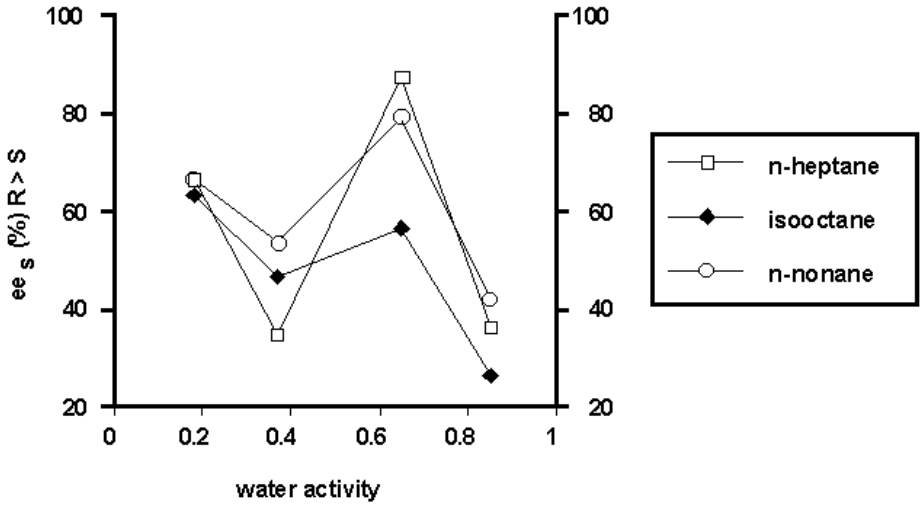


**Figure 7. Enantiomeric excess of the *Mucor javanicus* lipase-catalyzed reactions**

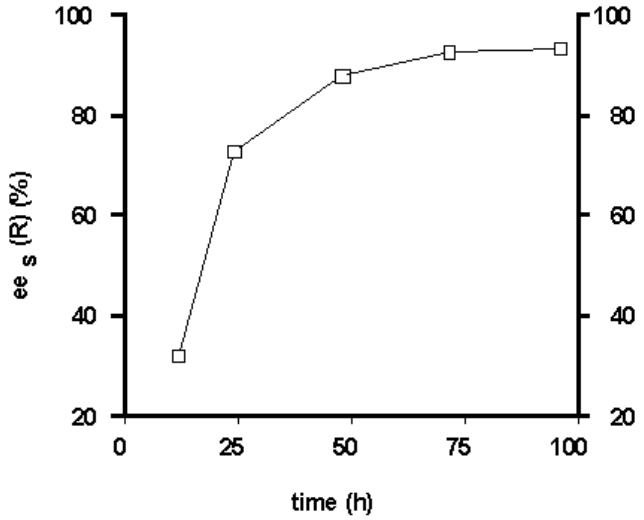


**Figure 8. Time-dependence of the enantiomeric excess of the R-enantiomer of flurbiprofen for the *Candida rugosa* lipase-catalyzed reaction in isooctane at 0.65 water activity.**

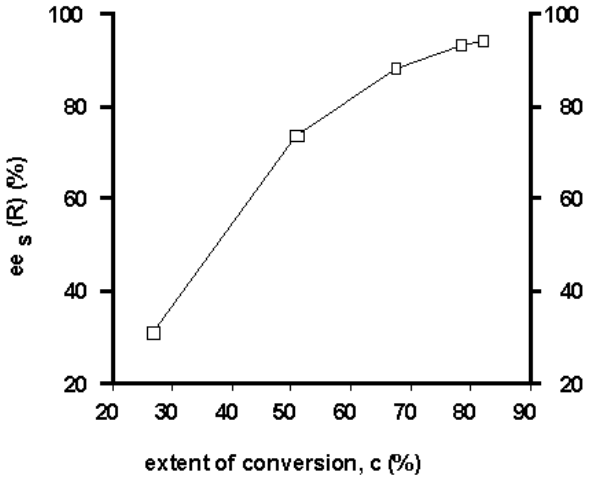


**Figure 9. Enantiomeric excess of the R-enantiomer of flurbiprofen as a function of the total substrate converted to product by the *Candida rugosa* lipase-catalyzed reaction in isooctane at 0.65 water activity.**

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