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Modelling the biokinetic resolution of diastereomers present in unequal initial amounts

Carla C. C. R. de Carvalho, Frederik van Keulen and M. Manuela R. da Fonseca*

Centro de Engenharia Biológica e Química, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal

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Abstract—The enantiomeric ratio (E) is commonly used to evaluate enzyme-catalysed kinetic resolutions. Chen et al. (1982) proposed a model for the enantiomeric ratio, which relates the extent of substrate conversion and the enantiomeric excess. The model, however, does not apply to cases where the substrate initially contains unequal amounts of enantiomers. This paper describes the adaptation of Chen's model to cases where the reagent consists of a mixture of diastereomers present in unequal initial amounts and gives product as a single diastereomer. Diastereomeric 'resolutions' of (+)-limonene-1,2-epoxide and of (−)-carveol were used to validate this model.

1. Introduction

Chiral building blocks, pharmaceutical or agrochemical compounds and food additives are commercially considered as isolated enantiomers only when the enantiomeric purity is very high (usually the enantiomeric excess has to be greater than 98%) Thus, efforts to obtain compounds in enantiomerically pure form and models for processes in which enantiopure compounds are produced are of increasing interest and importance.

The enantiomeric ratio (E) is commonly used to evaluate enzyme-catalysed kinetic resolutions. So far, the methods described in the literature to determine the enantiomeric ratio in enantioselective biocatalysis have mainly focused on the resolution of a racemic substrate[1–3] and to our knowledge, models describing the resolution of substrates that contain unequal initial amounts of enantiomers have not been developed as yet.

The model presented in this work is based on the model of Chen et al.,[1] and takes into consideration the fraction of each of the two diastereomers/isomers present in the initial substrate, and thus the initial diastereomeric excess. It is assumed that the diastereomeric selectivity of a biocatalyst may be described in a similar way to the enantioselectivity. A model correlating the diastereomeric excess of the substrates, the rate of conversion and the diastereomeric ratio was thus developed.

The model that is presented herein was experimentally validated in two case-studies involving the diastereoselective biotransformations of (+)-limonene-1,2-epoxide to limonene-1,2-diol and of (−)-carveol to carvone, both reactions being catalysed by whole cells of *Rhodococcus erythropolis* DCL14.

1.1. Model

If A_0 and B_0 represent the concentration of each of the isomers in the initial mixture and α and β the initial fraction of the fast (A) and slow (B) reacting isomers in an irreversible reaction with no inhibition by products, then:

$$A_0 = \frac{\alpha}{\beta} B_0 \tag{1}$$

and

$$A_0 = \frac{1}{x}(A_0 + B_0) \Leftrightarrow x = 1 + \frac{\beta}{\alpha} \tag{2}$$

$$B_0 = \frac{1}{y}(A_0 + B_0) \Leftrightarrow y = 1 + \frac{\alpha}{\beta} \tag{3}$$

where x and y are the reciprocal of the initial fractions of A and B, respectively.

The enantiomeric ratio (E) is defined as:

* Corresponding author. Tel.: +351-21-841 72 33; fax: +351-21-848 00 72; e-mail: mmrf@alfa.ist.utl.pt

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$$E=\frac{\ln\left(\frac{A}{A_0}\right)}{\ln\left(\frac{B}{B_0}\right)} \tag{4}$$

By applying this definition to the situation under study, in which both fast and slow reacting diastereomers produce the same product, the diastereomeric ratio (D) can be written as:

$$D=\frac{\ln\left(\frac{A}{\frac{1}{x}(A_0+B_0)}\right)}{\ln\left(\frac{B}{\frac{1}{y}(A_0+B_0)}\right)}=\frac{\ln\left[\frac{\frac{x}{2}(A+B)+\frac{x}{2}(A-B)}{A+B}\cdot\frac{A+B}{A_0+B_0}\right]}{\ln\left[\frac{\frac{y}{2}(A+B)+\frac{y}{2}(B-A)}{A+B}\cdot\frac{A+B}{A_0+B_0}\right]}=\frac{\ln\left[\frac{x}{2}\left(1+\frac{A-B}{A+B}\right)\cdot\frac{A+B}{A_0+B_0}\right]}{\ln\left[\frac{y}{2}\left(1+\frac{B-A}{A+B}\right)\cdot\frac{A+B}{A_0+B_0}\right]} \tag{5}$$

The extent of conversion, ξ, is given by

$$\xi=1-\frac{A+B}{A_0+B_0} \tag{6}$$

and the diastereomeric excess, de, defined by analogy with the enantiomeric excess, ee, as

$$de=\frac{B-A}{A+B} \tag{7}$$

The substitution of Eqs. (6) and (7) in Eq. (5) gives:

$$D=\frac{\ln\left[\frac{x}{2}(1-de)(1-\xi)\right]}{\ln\left[\frac{y}{2}(1+de)(1-\xi)\right]}=\frac{\ln\left[\frac{1}{2}\left(1+\frac{\beta}{\alpha}\right)(1-de)(1-\xi)\right]}{\ln\left[\frac{1}{2}\left(1+\frac{\alpha}{\beta}\right)(1+de)(1-\xi)\right]} \tag{8}$$

Rearrangement of Eq. (8) results in:

$$\xi=1-\left[\frac{\frac{x}{2}(1-de)}{\left(\frac{y}{2}\right)^D(1+de)^D}\right]^{\frac{1}{D-1}}=1-\left[\frac{\frac{1}{2}\left(1+\frac{\beta}{\alpha}\right)(1-de)}{\left(\frac{1}{2}\left(1+\frac{\alpha}{\beta}\right)\right)^D(1+de)^D}\right]^{\frac{1}{D-1}} \tag{9}$$

The initial fractions of the fast and slow reacting isomers can also be used for calculating the initial diastereomeric excess, de_0, defined as

$$de_0=\frac{B_0-A_0}{A_0+B_0}$$

In this case, Eqs. (8) and (9) can be expressed, respectively, as

$$D=\frac{\ln\left[\left(\frac{1-de}{1-de_0}\right)(1-\xi)\right]}{\ln\left[\left(\frac{1+de}{1+de_0}\right)(1-\xi)\right]} \tag{8'}$$

and

$$\xi=1-\left[\frac{\left(\frac{1-de}{1-de_0}\right)}{\left(\frac{1+de}{1+de_0}\right)^D}\right]^{\frac{1}{D-1}} \tag{9'}$$

As with the kinetic resolution of a racemic mixture, in this case the value of the diastereomeric ratio can also be estimated from the measurements of conversion and diastereomeric excess obtained during the course of the biotransformation. Chen et al.[1] proposed a model for recycling processes in which they considered that the resulting product, formed in the first step of the resolution (first cycle of the recycling process), may be transformed into the initial reagents. The next step of the recycling process would then start with a non-racemic mixture. This model correlates the extent of conversion with the enantiomeric ratio, the enantiomeric excess of the initial antipodal mixture, and the enantiomeric excess of the recycled product fraction. However, in our case 'the enantiomeric excess of the recycled product fraction' is zero (only one product) and the enantiomeric excess of the initial antipodal mixture is constant. Thus, if Chen's model were used, the enantiomeric ratio would depend on the conversion only. This is a further reason why Eq. (9) is useful in the estimation of de for the type of conversions addressed in the current study.

Fig. 1 shows several plots of D obtained by the resolution of Eq. (9) for different initial ratios of the diastereomers present in the mixture. These plots provide a useful overview of the relationships between de, ξ and D for a given α. It can be observed that for a D of 50, for example, when the initial ratio of the fast reactant is small, the reaction should be stopped at low degrees of conversion to attain a high de. On the other hand, when the initial amount of the fast reactant is high, it is necessary to have high degrees of conversion to obtain reasonable values of de, even for high values of D. The plots for high values of D are much closer to one another than their counterparts at low D values, i.e. a D of 100 is almost as effective as a $D=\infty$, but a significant difference to a D of 10 is observed. Nevertheless, even low D values can be exploited to obtain products with high enantiomeric/diastereomeric excess.

2. Results and discussion

2.1. Biotransformation of limonene-1,2-epoxide

R. erythropolis DCL14 cells contain a highly active limonene epoxide hydrolase (LEH) when grown on monoterpenes. According to van der Werf et al.,[4] the hydrolysis of (+)- and (−)-limonene-1,2-epoxide catalysed by the pure enzyme resulted in respectively (+)-(1*S*,2*S*,4*R*)- and (−)-(1*R*,2*R*,4*S*)-limonene-1,2-diol

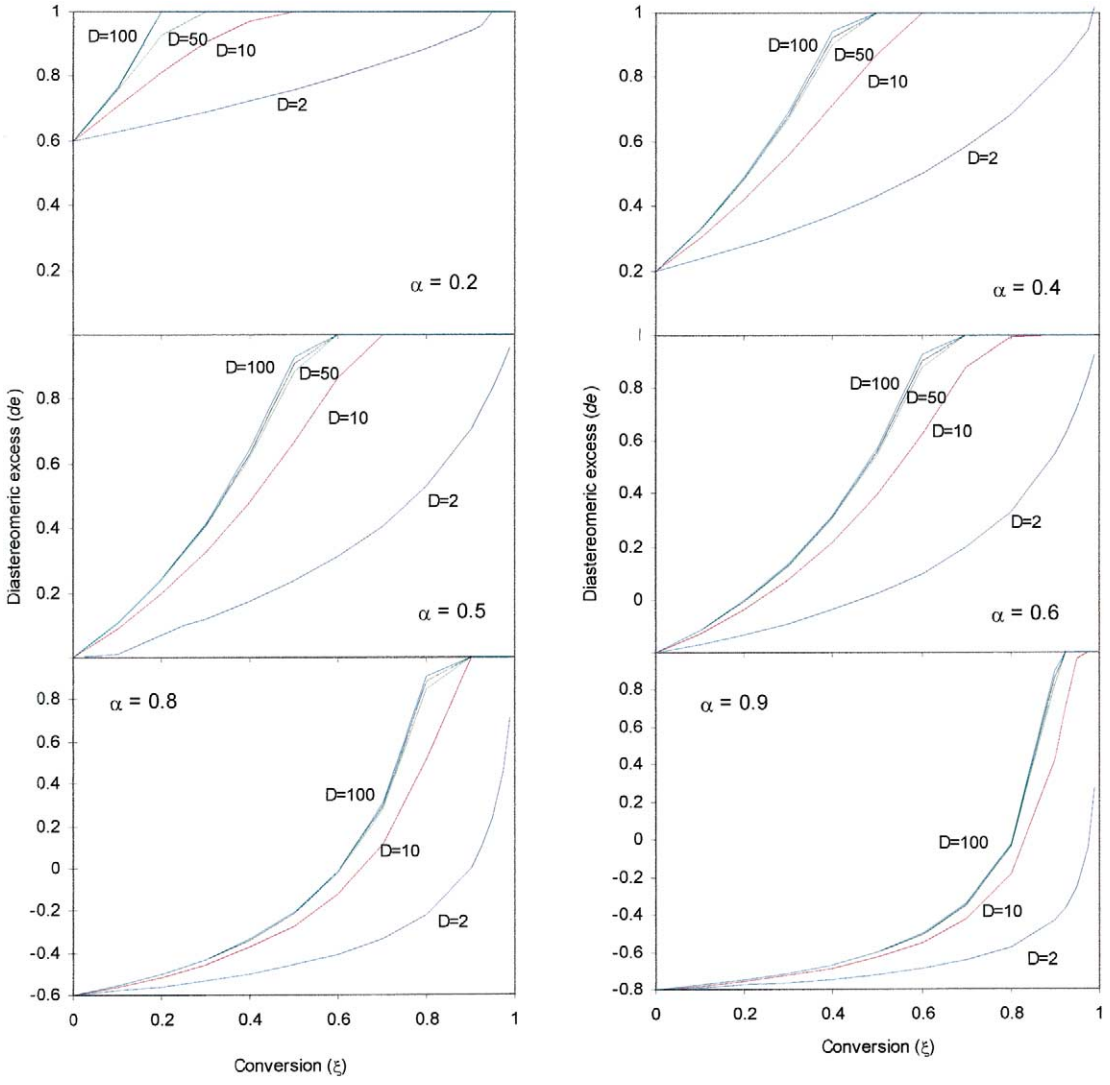

Figure 1. Diastereomeric excess versus degree of conversion, with the diastereomeric ratio (D) as parameter, for different relative initial amounts (α) of the fast reacting isomer.

as products. When LEH was added to a mixture of *cis*- and *trans*-limonene-1,2-epoxide, the sequential degradation of the (1*R*,2*S*)- and then the (1*S*,2*R*)-diastereomer was observed.[4]

In the present work, *R. erythropolis* cells were used to carry out the hydrolysis of (+)-limonene-1,2-epoxide (Fig. 2). The purpose of the reaction can be two-fold: (i) production of the diol from (+)-limonene-1,2-epoxide and (ii) diastereomeric resolution of mixtures of *cis*/*trans* epoxide.

By analogy with Eq. (7), we defined 'diastereomeric excess' as:

$$de = \frac{trans - cis}{trans + cis} \tag{10}$$

The (+)-limonene epoxide used in this work was a mixture of 42.2% of the *cis*- and 55.7% of the *trans*-epoxide. Substituting the values of α and β in Eqs. (2) and (3), we obtain 2.32 and 1.76 for x and y, respectively. The introduction of these values in Eq. (8) gives:

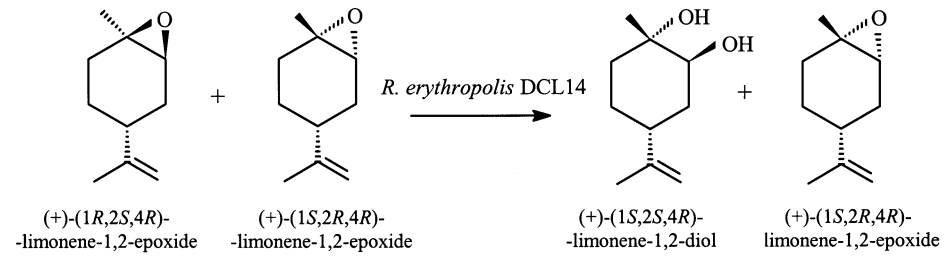

Figure 2. Conversion of (+)-limonene-1,2-epoxide by whole cells of *R. erythropolis* DCL14.

$$D=\frac{\ln[1.16\ (1-de)(1-\xi)]}{\ln[0.88\ (1+de)(1-\xi)]} \quad (11)$$

Rearrangement of Eq. (11) similar to that performed with Eq. (8) (which resulted in Eq. (9)) was used to estimate the values of D for the 'whole cell' biotransformation using experimental results on diastereomeric excess and degree of conversion. The data were obtained in two configurations of biphasic reactors: (i) mechanically stirred with direct-phase contact (at two initial substrate concentrations) and (ii) membrane reactor (Table 1). The values were fitted using the program TableCurve™2D from Jandel Scientific and confirmed by the Solver function of Microsoft Excel, version 7.0 for Windows 95.

Table 1. 'Resolution' selectivity of a mixture of *cis*- and *trans*-limonene-1,2-epoxide carried out by whole cells of *R. erythropolis* DCL14 with activity in limonene epoxide hydrolase, evaluated by the diastereomeric ratio

Reactor	D
Direct-phase contact, 2.5 mM	86.1
Direct-phase contact, 50 mM	86.4
Membrane, 75 mM	86.5

With the mean value of D from Table 1, Eq. (11) was solved using the Solver function of Microsoft Excel, version 7.0. The diastereomeric excess predicted by the model is plotted in Fig. 3 as a function of substrate conversion.

When the degree of conversion of limonene-1,2-epoxide reached the value of 0.43, the *de* was higher than 0.99. This is an indication that this process is suitable for the production of *trans*-limonene epoxide (Fig. 3). The model was able to fit well with the experimental results.

2.2. Biotransformation of carveol

According to van der Werf et al.,[5] *R. erythropolis* DCL14 cells contain a carveol dehydrogenase (CDH) which allows for the stereoselective oxidation of (+)-*cis*-carveol to (+)-carvone and of (−)-*trans*-carveol to (−)-carvone. The conversion of the respective diastereomers by CDH was not observed by these authors.

When a mixture of diastereomeric, *cis*- and *trans*-(−)-carveol, was subjected to biotransformation with whole cells, the (−)-*trans*-carveol was converted to (−)-carvone. Two products were obtained: (i) (−)-carvone and (ii) diastereomerically resolved (−)-*cis*-carveol[6] (Fig. 4).

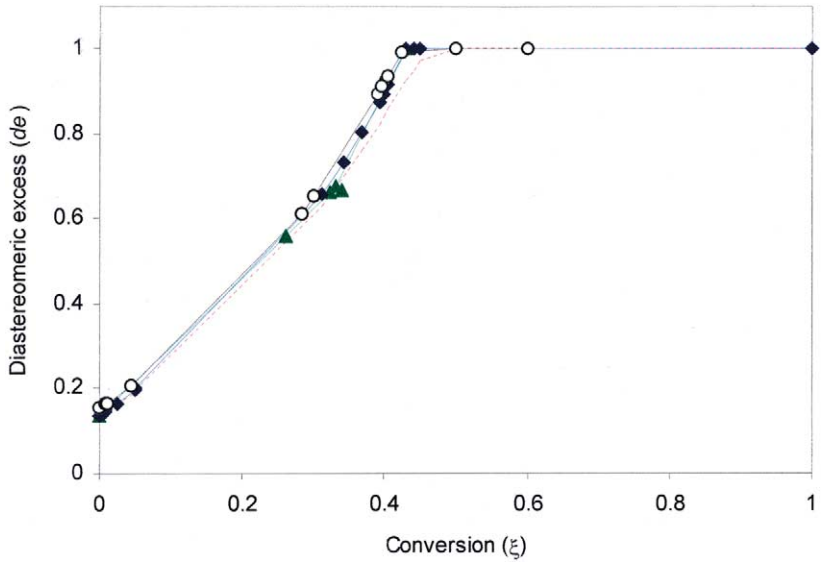

Figure 3. Diastereomeric excess of *trans*-limonene epoxide (▲ and ◆—experimental values obtained in a direct-phase contact reactor at an initial epoxide concentration of 2.5 and 50 mM, respectively; ○—experimental values obtained in a membrane reactor; --- model, obtained by resolution of Eq. (11) for $D=86.3$).

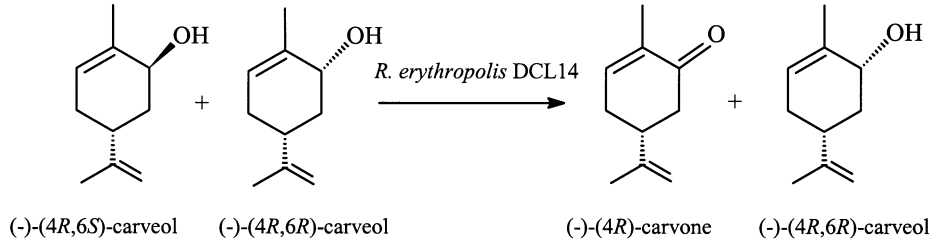

Figure 4. Conversion of (−)-carveol by whole cells of *R. erythropolis* DCL14.

However, we could detect the transformation of the *cis*-diastereomer when the concentration of *trans*-carveol reached rather low levels.[7]

The (−)-carveol used in this experiments was a mixture of 38% (−)-*cis*- and 62% (−)-*trans*-carveol. The amount of the fast reacting diastereomer, (−)-*trans*, being higher than that of the slow reacting, gives rise to negative diastereomeric excess values before the two quantities become equal. Straathof and Jongejan[3] considered *ee* as being always positive, i.e. they included a modulus in its definition. However, in the present case, the amount of the fast reacting diastereomer, which is higher in the beginning, decreases during the biotransformation and is even smaller than the amount of the slow reacting isomer at the end. If we introduced the modulus in the *de* definition, the curves of *de* versus degree of conversion would take a less common shape. The diastereomeric excess was thus calculated as:

$$de = \frac{cis - trans}{trans + cis} \tag{12}$$

Substituting the values of α and β in Eqs. (2) and (3), the x and y values obtained are 1.61 and 2.63, respectively. Substituting for x and y in Eq. (8) gives:

$$D = \frac{\ln[0.81\ (1-de)(1-\xi)]}{\ln[1.32\ (1+de)(1-\xi)]} \tag{13}$$

CDH activity and co-factor dependence vary with the carbon source used for *R. eryhtropolis* growth.[5] The values of D obtained (using TableCurve™2D from Jandel Scientific) for the CDH resolutions with cells grown on limonene only, cyclohexanol only and on both substrates were 67.0, 67.0 and 67.2, respectively. Biotransformation runs using cells grown under the above three sets of conditions were carried out to assess the effect on selectivity of cofactor dependence. No significant differences in CDH selectivity were observed (Fig. 5) and the experimental values correlate well with the model (Fig. 5).

When *trans*-carveol consumption was almost complete (at ca. 96% conversion), degradation of *cis*-carveol started to occur. From this time onwards, the *cis*-isomer was consumed at a higher rate than the remaining *trans*-carveol, although at a much lower rate than the initial consumption of *trans*-carveol (0.789 and 32.77 nmol $_{carvone}$/min mg_{prot}, respectively). At this point, the *de* obtained was only 0.9 (Fig. 5), although the isomeric ratio during this period was 67.1 (mean value for the three situations). According to Eq. (12), the value of *de* decreases as the *cis*-diastereomer is converted. If we take into consideration the fact that the slow reacting diastereomer is now *trans*-carveol, then the value of *de* will increase, but all the values will be negative. At the switch point there were 4.9 and 95.1% of *trans*- and *cis*-carveol, respectively. Hence the equation used was:

$$D = \frac{\ln[0.526\ (1-de)(1-\xi)]}{\ln[10.24\ (1+de)(1-\xi)]} \tag{14}$$

During this later period, the values of D, calculated using the software referred to above, were 2.45, 1.95 and 2.00 for the CDH resolutions with cells grown on limonene, cyclohexanol and on both substrates, respec-

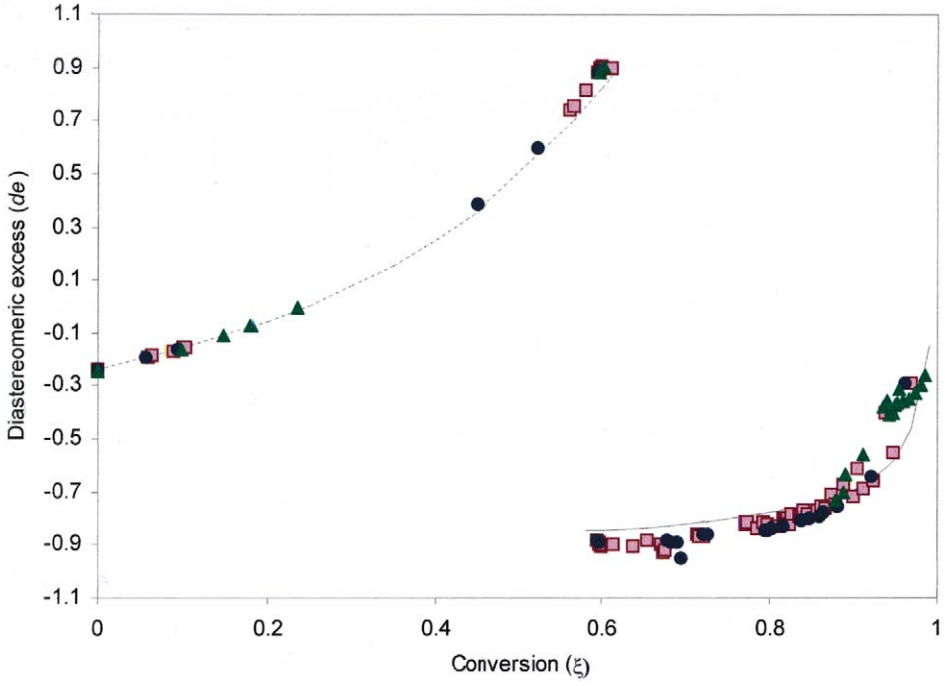

Figure 5. Diastereomeric excess during the conversion of (−)-carveol. For conversion degrees lower than 0.6 there is a diastereomeric excess of *cis*-carveol. For conversion degrees higher than 0.6 there is a diastereomeric excess of *trans*-carveol (■, ● and ▲—experimental values obtained with cells grown on limonene+cyclohexanol, cyclohexanol and limonene, respectively, as carbon source; direct-phase contact reactor with a carveol concentration of 50 mM; --- model, for $D = 67.1$; ··· model, for $D = 2.1$).

tively. The diastereomeric ratio was thus much lower than that obtained during the fast *trans*-carveol consumption period. Model fitting to the experimental data was not as good as in the previous examples.

In a 500 mL mechanically stirred reactor, complete consumption of *trans*-carveol could be achieved.[8] By adding substrate every time the organic phase was depleted in *trans*-carveol, several 'initial' *trans*/*cis* ratios were obtained. Fig. 6 shows how well the model fits the data obtained at the different 'initial' substrate ratios.

3. Conclusions

There are large numbers of methods available for the accurate determination of E values.[2] However, non-racemic substrates and isomeric/diastereomeric resolutions with a single product obtained from both diastereomers have not, to our knowledge, been addressed. The extension to Chen's model, which is presented in this work is intended at reducing this gap.

Two case-studies were discussed, regarding the diastereomeric resolution of (+)-limonene-1,2-epoxide and of (−)-carveol, both catalysed by whole cells of *R. erythropolis* DCL14. It was shown that the proposed extension to the model satisfactorily describes this type of kinetic 'resolutions'. These two sets of experimental data thus validate the model.

4. Experimental

Microorganism: *R. erythropolis* DCL14 was supplied by the Division of Industrial Microbiology of the Wageningen Agricultural University, Wageningen, The Netherlands, in the context of the European Project BIO4-CT95-0049.

Chemicals: The terpenes used were (*R*)-(+)-limonene (97%), (+)-limonene oxide (97%), (−)-carveol (97%) and (*R*)-(−)-carvone (98%), from Aldrich Chemicals.

4.1. Limonene-1,2-epoxide biotransformation

Growth: Cells were grown at 30°C and 400 rpm in a 2.0 L fermenter containing 1.5 L of mineral medium.[9] The growth substrate, (*R*)-(+)-limonene, was continuously fed via the gas phase by bubbling the inlet air, at 100 ml/min, through the sintered glass sparger of a glass bottle containing limonene. Growth was followed by measuring the O.D. (600 nm); when the value was higher than 1.5, 2/3 of the fermentation broth was removed and cells were harvested by centrifugation (7000 rpm, 10 min). The fermenter was refilled with fresh medium.

Reactions: Assays were carried out in a 500 mL reactor (containing 300 mL of 50 mM phosphate buffer, pH 7.0, 60 ml of organic phase, defined concentrations of epoxide and about 50 mg, d.w., of cells) and in a membrane reactor (600 mL of aqueous phase; the substrate was recirculated through a silicone tube with 149.4 cm^2, acting as a membrane). Reactors were mechanically stirred at 200 rpm and operated at room temperature. Reactions were followed by monitoring the diol accumulation in the aqueous phase and the substrate consumption in the organic phase.

Analysis: At regular intervals the aqueous phase was sampled to follow diol production. The diol in the samples was extracted with ethyl acetate (1:1), which was analysed by gas chromatography (GC) on a Hewlett Packard 5890 gas chromatograph with a FID detector, connected to a HP3394 integrator. The capillary column was a SGE HT5, 25 m in length and with internal and external diameters of 0.22 and 0.33 mm, respectively. The oven temperature was 150°C and the

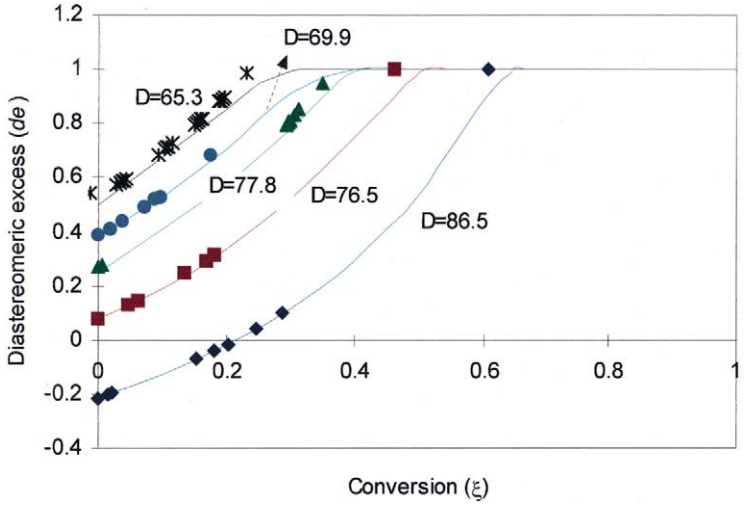

Figure 6. Diastereomeric excess observed during conversion of (−)-carveol by whole cells of *R. erythropolis* DCL14 in a direct contact stirred reactor. Values of D calculated by the model for each curve.

temperature of the injector and detector was 250°C. Organic-phase samples were taken whenever it was important to follow epoxide consumption. These were injected without any previous treatment and analysed for their epoxide content. The oven temperature of the gas chromatograph was 150°C and that of the injector and detector was 250°C. To separate the epoxide isomers the oven temperature was 90°C, while the injector and detector were at 250°C.

4.2. Carveol biotransformation

Growth: Cells were grown at 28°C and 400 rpm in a 2.0 L fermenter containing 1.5 L of mineral medium.[9] The inlet air flow was 200 mL/min. Limonene was supplied through the air stream, as previously described. For cells grown on cyclohexanol, a solution of cyclohexanol (0.2 mM) was added at a flow rate of 6.3 mL/h. When cells were grown in both limonene and cyclohexanol, limonene was added through the air stream and a solution of cyclohexanol was added to the fermenter, as described for the single carbon source situations. In all cases, cells where harvested when the O.D. value was higher than 4.

Reactions: Activity assays were carried out, at least in duplicate, in 60 mL flasks closed with rubber bungs, containing 20 mL of 50 mM phosphate buffer (pH 7.0), 20 mL of organic phase and defined substrate concentrations. After the addition of a concentrated suspension of whole cells of *R. erythropolis* DCL14, the flasks were incubated at 28°C and 200 rpm. Reactions were followed by monitoring carvone accumulation in the organic phase.

Mechanically stirred reactor: Assays were carried out in a 500 mL reactor containing 300 mL of 50 mM phosphate buffer pH 7.0, 60 mL of organic solvent (*n*-dodecane), 50 mM of (−)-carveol (referred to the organic phase) and an initial O.D. (measured at 600 nm) of 0.8. The reactor was mechanically stirred and operated at room temperature. Every time the organic phase was depleted in *trans*-carveol, 0.49 mL of (−)-carveol was added.

Analysis: At regular intervals the organic phase was sampled and the carveol and carvone were subsequently analysed by gas chromatography (GC) on a Hewlett Packard 5890 gas chromatograph with a HP3394 integrator, without any previous treatment. The capillary column was a SGE HT5, 25 m in length and with internal and external diameters of 0.22 and 0.33 mm, respectively. The oven temperature was 140°C and the injector and detector were at 200 and 250°C, respectively.

Error analysis: The error associated with the GC analysis of samples of the organic phase was ±6% and that of the aqueous phase, extracted with ethyl acetate prior to injections, was ±8%. The errors were calculated based on the standard deviation and sample mean of seven repeated injections and are quoted for a confidence interval of 95%. Biomass concentration measurements (O.D.) had an associated error of ±8% based on the standard deviation and sample mean of eight repeated samples, quoted for a confidence interval of 95%.

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