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# Kinetic Analysis and Modeling of the Liquid–Liquid Conversion of Emulsified di-Rhamnolipids by Naringinase From *Penicillium decumbens*

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**ABSTRACT:** The enzymatic conversion of an aggregates-building substrate was kinetically analyzed and a model was applied for the prediction of reaction-time courses. An L-rhamnose molecule from a di-rhamnolipid is cleaved by Naringinase from *Penicillium decumbens* leading to a mono-rhamnolipid. Optimal reaction rates were found when both, substrate and product build large co-aggregates in a slightly acidic aqueous phase. On the other hand, reaction rates were independent of initial di-rhamnolipid concentration and this was interpreted by assuming that the reaction occurs in the aqueous phase according to Michaelis–Menten kinetics in combination with competitive L-rhamnose inhibition. Rhamnolipids were therefore assumed to be highly concentrated in aggregates, a second liquid phase, whereas diffusive rhamnolipid transport from and to the aqueous phase occurs due to the enzymatic reaction. Furthermore, ideal surfactant mixing between di- and mono-rhamnolipid was assumed for interpretation of the negative effect of the last on the reaction rate. A model was created that describes the system accordingly. The comparison of the experimental data, were in excellent agreement with the predicted values. The findings of this study may beneficially be adapted for any bioconversions involving aggregate-forming substrate and/or product being catalyzed by hydrophilic enzymes.

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**KEYWORDS:** rhamnolipids; liquid–liquid biocatalysis; Naringinase; micellar substrates

## Introduction

Rhamnolipids (RL) are amphiphilic compounds produced by, for example, *Pseudomonas* sp. from hydrophobic carbon sources as, for example, vegetable oils. Because of their biodegradability, high tensioactive properties and production from renewal and inexpensive raw materials they are of special interest as an alternative to chemically synthesized surfactants. Besides their use as detergents, RL also have potential industrial applications in food, cosmetic, pharmaceuticals, as well as in bioremediation of pollutants (Banat et al., 2000; Mulligan, 2005; Nitschke et al., 2005). RL are produced as an extracellular mixture of different species, which composition strongly varies according to process conditions (Lang and Trowitzsch-Kienast, 2002; Nitschke et al., 2005). The main species commonly encountered are often termed di-rhamnolipid (di-RL) and mono-rhamnolipid (mono-RL) (see Fig. 1). RL are very potent biosurfactants showing decreased surface tension of water at very low concentrations (26 mg $L^{-1}$) (Lang and Trowitzsch-Kienast, 2002; Ozdemir et al., 2004). Although having similar critical micelle concentration values (*cmc*), mono-RL shows higher surface and interfacial activity than di-RL at concentrations below the *cmc*. This property is attributed to the more favorable hydrophilic/hydrophobic balance of mono-RL molecules (Ozdemir et al., 2004).

The interest of RL was often targeted to the production of L-rhamnose and therefore RL were chemically or enzymatically hydrolyzed for obtaining this desoxy sugar (Giani et al., 1993; Linhardt et al., 1989; Meiwes et al., 1997; Mixich et al., 1996; Trummler et al., 2003). L-Rhamnose is mainly used as a starting material for the production of the flavor agent Furaneol® (Lang and Trowitzsch-Kienast, 2002; Trummler et al., 2003). Enzymatic hydrolysis of di-RL, as displayed in Figure 1, could be a conclusive method for the simultaneous production of pure mono-RL as well as L-rhamnose.

Therefore, the objective of this study was the characterization and modeling of the hydrolysis kinetics of di-RL for the production of mono-RL and L-rhamnose by Naringinase. Besides, the subsequent reaction with formation of L-rhamnose

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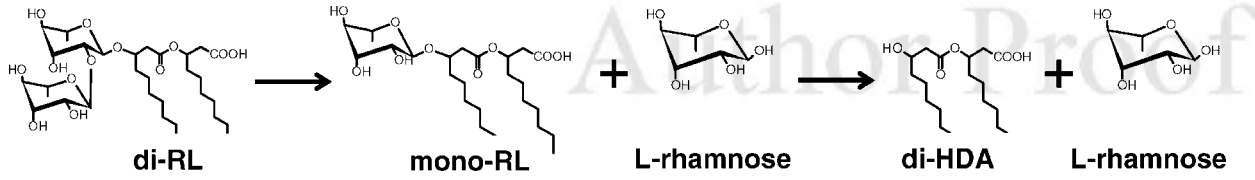


**Figure 1.** Hydrolysis of di-rhamnolipid and mono-rhamnolipid.

and 3-(3-hydroxydecyloxy)decanoic acid (di-HDA) from mono-RL was also investigated. To understand the kinetics of an enzymatic conversion of aggregates-building substrates such as RL, a kinetic analysis for describing reaction-time courses was set-up.

### Reaction Kinetics Modeling

At concentrations above the *cmc* RL build micelles. One of the approaches of thermodynamic treatments of micelle formation considers the micelles to form a separate phase at the *cmc* (Attwood and Florence, 1983; Holland and Rubingh, 1992). In account of this RL were assumed to build a highly RL-concentrated liquid phase termed as the micelle-phase. On the other hand, an enzyme attack was assumed to be only possible for dissolved molecules or monomers. Therefore, a diffusive mass transport of di-RL from the micelle-phase to the aqueous phase has to occur due to the decomposition of this species by the enzyme. Equally, a diffusive mass transport of the emerging reaction product mono-RL occurs in the opposite direction whereas the product L-rhamnose accumulates in the aqueous phase. The influence of liquid–liquid mass transfer on the overall reaction rate was checked according to Levenspiel (1999) and Straathof (2003).

The overall RL content was measured as the sum of aqueous and micelle-phase concentration, represented as $C^{T}_{di-RL}$ and $C^{T}_{mono-RL}$. Thereby, mole fractions $X^{T}_{di-RL}$ and $X^{T}_{mono-RL}$ were calculated. Assuming that RL is concentrated in the micelle-phase and under steady-state condition of the aqueous phase (Straathof, 2003) the mass balance for mono-RL corresponds to:

$$C^{T}_{RL}\frac{dX^{T}_{mono-RL}}{dt} = -r\frac{V_{aq}}{V_{T}} \quad (1)$$

In Equation (1), $r$ represents the reaction rate. It was assumed that (1) the RL diffusion into and from the aqueous phase is not accelerated by the enzymatic reaction, (2) no reaction takes place in the interface film, and (3) constant volumes of aqueous and micelle phases during reaction time. The reaction rate was described by the Michaelis–Menten kinetic with competitive product inhibition by L-rhamnose. The Michaelis–Menten constant $K_m$ was assumed to be much higher than the aqueous di-RL concentration since the last was determined to be rather low (see Results Section) and thus unlikely as being as high as the $K_m$ value considering typical $K_m$ values for other substrates (Romero et al., 1985). Therewith, $r$ equals:

$$r = \frac{-A_s\rho_E C^{aq}_{di-RL}}{K_m\left(1+\frac{C_{Rha}}{K_I}\right)} \quad (2)$$

Combining Equation (2) with the di-RL mass transfer rate $\phi_{di-RL}$ an expression for $C^{aq}_{di-RL}$ was derived:

$$C^{aq}_{di-RL} = \frac{k_{L,di-RL}aC^{aq,eq}_{di-RL}\left(1+\frac{C_{Rha}}{K_I}\right)}{\frac{A_s}{K_m}\rho_E + k_{L,di-RL}a\left(1+\frac{C_{Rha}}{K_I}\right)} \quad (3)$$

Considering the separate phase approach for characterizing mixed surfactants systems and assuming ideal mixing, the monomer concentration of di-RL in equilibrium and its total mole fraction were related according to Holland and Rubingh (1992) and Milioto (2006):

$$C^{aq,eq}_{di-RL} = X^{T}_{di-RL}\,cmc_{di-RL} \quad \text{for} \quad C^{T}_{RL} \gg cmc_{di-RL} \quad (4)$$

Furthermore, $C_{Rha}$ was calculated as the difference of the initial total di-RL concentration and the total di-RL concentration at every time. For integration of Equation (1), the following boundary condition was used:

$$t = 0; \quad X^{T}_{mono-RL} = X^{T}_{mono-RL,0}$$

For low RL total concentrations (<0.01 M) the ratio of aqueous to total volume of Equation (1) was assumed to be unity. For numerical integration of Equation (1) by the Runge–Kutta method and fitting to experimental data the program ModelMaker Version 3.0.3 (Cherwell Scientific Publishing Ltd., Oxford, UK) was used.

## Materials and Methods

### Materials

Naringinase (N-1385; Lot N° 110K16471; 511 U g$^{-1}$ L-rhamnosidase activity, 55 U g$^{-1}$ β-glucosidase activity)

from *Penicillium decumbens* was purchased from Sigma–Aldrich (Steinheim, Germany). Pure crystalline di-RL was by courtesy of Hoechst AG (Frankfurt, Germany) (97%). The model substrate *p*-nitro-phenyl-rhamnoside (pnpR) for assaying α-rhamnosidase activity was obtained from Extrasynthese (Genay, France). Highly pure standards of mono-RL and di-HDA were prepared by enzymatic hydrolysis of 1 and 2 g di-RL, respectively, according to Trummler et al. (2003). After production and solvent extraction, mono-RL and di-HDA were further purified by adsorption chromatography: Silica gel (60 DM 0.04–0.063 mm) was used as stationary phase and a system methanol–chloroform as mobile phase (ratio 15:85 for mono-RL and 5:95 for di-HDA). After evaporation of the mobile phase, silica gel impurities were removed by extraction with water from the re-dissolved products in ethyl acetate for mono-RL and in hexane for di-HDA. After drying, the organic phase was evaporated under high vacuum. 300 mg mono-RL and 454 mg di-HDA were obtained as honey-like substances. HPLC measurements of the products gave single peaks and elemental analysis was in accordance with theoretical values. mono-RL used as substrate for biotransformation assays was also produced by enzymatic hydrolysis of di-RL without further purification (condition: pH 4.5, temperature 57°C, with free Naringinase). All other reagents, chemicals and co-solvents were of analytical grade.

## Activity Assays With Rhamnolipids

di-RL biotransformations were initiated by addition of a Naringinase solution to a temperate di-RL emulsion in a ratio 1–20 (see Table I for concentration settings). Under thermo-stated conditions and shaking in a thermo-bloc unit (Thermomixer comfort, Eppendorf AG) 500 μl samples were withdrawn at different times and the reaction was stopped by acidification with 50 μL 1 M phosphoric acid followed by RL extraction in 500 μL ethyl acetate. After centrifugation, the ethyl acetate phase was sampled and evaporated at 60°C over 1 h and the RL was re-dissolved in acetonitrile for analysis. di-RL emulsions were prepared by adding a buffer solution to a weighted amount of RL followed by equilibration at the desired temperature. Following 0.1 M buffer solutions were used: sodium formate for pH 2.5–3.5, sodium acetate for pH 4.0–5.5 and sodium phosphate for pH 6.0–6.5. Table I shows experimental conditions of different arrays of biotransformations carried out for optimization and kinetic studies.

For bioconversion with organic solvents, samples were diluted with buffer before ethyl acetate extraction to avoid impairment of RL extraction due to co-solvent addition. Shaking speed was 1,400 $min^{-1}$ for pH and temperature-curve setups and 700 $min^{-1}$ for all other experiments. Initial volumetric activities were calculated as the derivative at time zero of mono-RL mole fraction-time curves multiplied by the total nominal RL concentration. Since a linear time course could not be reached due to the experimental constraint of under-saturation conditions ($C^{aq}_{di-RL} \ll K_m$), experimental mole fraction-time values were fitted to the following equation:

$$X^{T}_{mono-RL} = b(1 - e^{-ct}) \quad (5)$$

The program SigmaPlot Version 9.01 (Systat software, Inc., 2004) was used for the fitting procedure. One unit was defined as the enzyme amount that converts 1 μmol mono-RL from di-RL in 1 min at specified conditions of temperature and pH.

## Activity Assays With pnpR

Activities of Naringinase solutions were always checked with pnpR before di-RL biotransformation: An enzyme solution was added to an 8 mM pnpR solution in 0.1 M sodium acetate buffer pH 5.5, into a plastic cuvette, at 60°C. The

**Table I.** Experimental conditions for optimization and kinetic studies of RL biotransformation.

| Array of biotransformations | pH | $T$ (°C) | $C^{T}_{di-RL,0}$ [a] (mM) | $C^{T}_{mono-RL,0}$ [a] (mM) | $\rho_E$ [b] ($mg\,L^{-1}$) | $R_{E/S}$ [c] (%) |
|---|---|---|---|---|---|---|
| Temperature-curve (Fig. 2) | 5.5 | 40–80 | 1 | 0 | 6 | 0.92 |
| pH-curve (Fig. 2) | 2.5–6.5 | 60 | 1 | 0 | 6 | 0.92 |
| Co-solvents assays (Table II) | 4.5 | 50 | 5 | 0 | 26 | 0.80 |
| Ethanol assays (Fig. 3) | 4.5 | 60 | 10 | 0 | 26 | 0.40 |
| Enzyme assays (Figs. 4A and 6A) | 4.5 | 60 | 10 | 0 | 260–6.5 | 4.0–0.10 |
| Substrate assays (Fig. 4B and 6B) | 4.5 | 60 | 0.1–10 | 0 | 4.9 | 7.5–0.075 |
| Rhamnose assays (Fig. 4C) | 4.5 | 60 | 3 | 0 | 4.9 | 0.25 |
| Mole fraction assays (Fig. 6C) | 4.5 | 60 | 3 | 0–9 | 4.9 | 0.25 |
| | 4.5 | 60 | 91 | 0 | 325 | 0.50 |
| High-substrate assays (Fig. 6D) | 4.5 | 60 | 47 | 0 | 4.9 | 0.015 |
| | 4.5 | 60 | 24 | 0 | 4.9 | 0.030 |
| mono-RL-conversions (Fig. 7) | 4.5 | 60 | 0 | 10 | 0.5 | 7.7 |
| | 4.5 | 60 | 10 | 0 | 0.5 | 9.9 |

[a]Initial total di-RL and mono-RL concentrations, respectively.
[b]Enzyme concentration.
[c]Mass ratio enzyme to substrate.

increase of *p*-nitrophenolate (pnp) concentration was followed by monitoring the absorption at 400 nm (extinction coefficient for pnp at pH 5.5 and 60°C: 1.2 l $mmol^{-1}$ $cm^{-1}$) during 5 min reaction in a photometer (Amersham Biosciences, Uppsala, Sweden) equipped with a heated cell changer and coupled to the software Swift II reaction kinetics (Biochrom Ltd., Cambridge, UK). For pnpR activity test with ethanol as co-solvent (Fig. 3), a 2 mM pnpR solution in sodium acetate buffer pH 4.5 and ethanol (0–50%) with and without addition of an RL solution (end concentration 10 mM) was heated at 60°C. Enzyme solution was added and activity was assayed according to Romero et al. (1985): At different times, samples were withdrawn and 100 μL were added into a cuvette with 1.5 mL 0.1 M sodium hydroxide and the absorption was immediately measured at 400 nm (Extinction coefficient for pnp at pH 12 and room temperature: 18.9 l $mmol^{-1}$ $cm^{-1}$).

### Determination of the Solubility, *cmc* and p$K_a$-Value of Rhamnolipids

di-RL emulsions (0.5–10 mM) prepared in 0.1 M sodium acetate buffer pH 4.5 and equilibrated for 3 h at 60°C under shaking were let to stand at 60°C for 2 days for complete sedimentation of the micelle-phase. Then, samples of the aqueous phase were taken and treated as described above for HPLC analysis. Emulsion mixtures with different ratios di-RL to mono-RL were prepared by carrying out biotransformations of di-RL (10 mM di-RL, 65 mg $L^{-1}$ enzyme concentration, 60°C and pH 4.5), which were stopped at different times before reaction completion. After analysis of the di-RL to mono-RL proportion, the ethyl acetate phase was evaporated and the RL emulsified in 0.1 M sodium acetate buffer pH 4.5 (10 mM final RL concentration). After equilibration at 60°C under shaking, emulsions were centrifuged (Avanti™ J-30I, Beckman Coulter™, CA[Q1]) at 75,600*g* and 25°C for 1 h. Samples of the aqueous phase were taken and treated as described above for HPLC analysis. The superficial tension of a set of di-RL solutions at defined pH and increasing concentrations was measured with a tensiomenter (Digital-tensiometer K10, Krüss, Hamburg, Germany) using the plate method and the *cmc* was defined as the concentration up to non-further decrease of superficial tension was observed. Determination of di-RL acidic constant ($K_a$) was determined potentiometrically by back-titration of a basic 5 mM di-RL solution with 0.1 M hydrochloric acid.

### Rhamnolipids Analytics

RL were analyzed by an HPLC device coupled to a UV detector (1100 Series, Agilent, Santa Clara[Q2]) according to Schenk et al. (1995): RL were pre-derivatized into esters of bromophenacylbromide (Fluka, Steinheim, Germany) in acetonitrile over 1.5 h at 60°C and applied to a RP-18 column (Supelcosil LC-18)(150 mm × 4.6 mm, 5 μm silica gel) thermo-stated at 25°C. Then, RL-bromophenacyl-esters were eluted at a flow rate of 0.8 mL $min^{-1}$ with a linear gradient acetonitrile(Acn)–water (0 min: 70% Acn, 4 min: 70% Acn, 14 min: 100% Acn, 28 min: 100% Acn, 33 min: 70% Acn; 38 min: 70% Acn) and detected at 265 nm. Calibration curves of di-RL, mono-RL and di-HDA were carried out in the range 0.1–1 mM with the same preparation protocol as for the biotransformation samples (acidification and extraction from a buffer solution). RL were also qualitatively analyzed by thin layer chromatography with a methanol–chloroform solution in a ratio 15:85 as mobile phase. RL spots in the plates were detected by immersion into a ammonium molibdate/cerium sulphate acidic solution (0.42% (w/v) ammonium molibdate, 0.2% (w/v) cerium(IV) sulphate and 6.2% (v/v) sulfuric acid) and after heating at 105°C.

## Results

### Selection of Reaction Temperature and pH

Figure 2 shows the influence of temperature and pH on the enzyme activity for di-RL conversion. A linear correlation was observed when plotting specific activities till 70°C in an Arrhenius array ($R^2 = 0.99$), and the activation energy of the reaction was therewith estimated to be $54 \pm 3$ kJ $mol^{-1}$. A temperature of 60°C was chosen for further experiments, where enzyme half-life values larger than 100 h were obtained (Magario et al., 2008a). A wide pH optimum

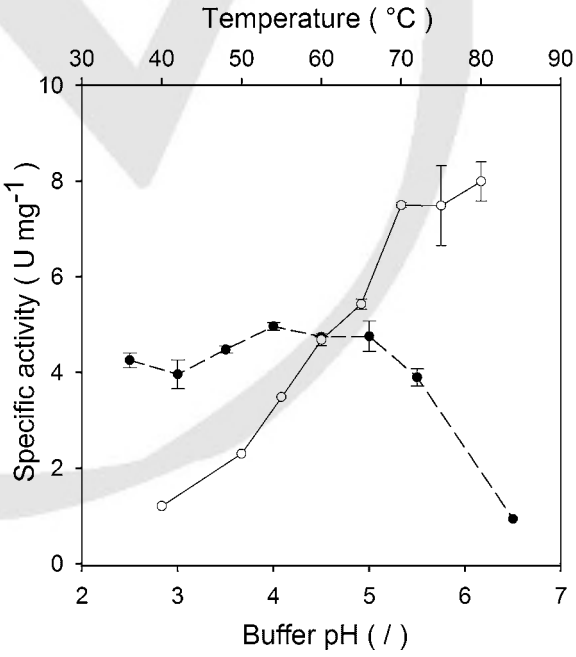


**Figure 2.** Influence of temperature (○) and pH (●) on the enzyme activity for di-RL-conversion.

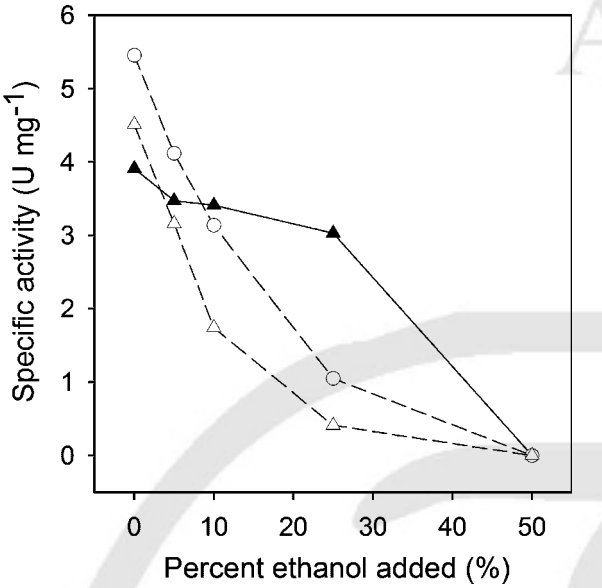


**Figure 3.** Effect of ethanol addition on enzyme activity. Activity measured with: di-RL (▲), pnpR (○), and pnpR in the presence of RL micelles (△).

between 4.0 and 5.0 is observed, which is in agreement with the optimum pH for stability (Magario et al., 2008a).

Measurements of *cmc* of di-RL at 61°C resulted in 0.08 mM at pH 7.0 and 0.03 at pH 4.5 whereas *cmc* of di-RL at pH 4.5 and 34°C resulted in 0.03 mM. These values indicate that the *cmc* increases with increasing pH however being not influenced by the temperature. This *cmc* tendency with pH was already obtained by Ozdemir et al. (2004) and Syldatk et al. (1985). However, as observed in Figure 2 enzyme activities may be more influenced by the optimal catalytic activity of Naringinase at acidic pH rather than by the higher availability of di-RL monomers at higher pHs.

At acidic pH values di-RL molecules exist mainly as suspended hydrated crystals. These crystals were solubilized at pH higher than 5. The acidic constant of di-RL was determined to be $7.6\ 10^{-7}$ ($pK_a$ 6.12). Thus, a relatively low ionization grade was enough to solubilize the non-protonated form of di-RL. On the other hand, when suspensions of di-RL at pH 4.5 were heated above approximately 50°C, solid di-RL crystals became a separate liquid phase building a turbid and unstable emulsion. This liquid rhamnolipid phase, the micelle-phase, may correspond to the formation of liquid crystals or large aggregates. This emulsion became a clear solution with increasing pH. This observation is in agreement with the fact that the size of the RL micelle-phase increases with decreasing pH (Champion et al., 1995; Ishigami et al., 1987; Lebron-Paler et al., 2006). Kinetics analysis was further conducted at pH 4.5 taking advantage of maximal reaction rates and the existence of a two-phase system, which simplifies product recovery by decantation or centrifugation of the micelle-phase.

## Conversions With Co-Solvent Addition

In order to observe whether the addition of water-soluble solvents increase reaction rates due to an increase in the di-rhamnolipid monomer concentration, different alcohol and non-alcohol type co-solvents were tested. Table II lists specific enzyme activities, mono-RL mole fraction reached after 4 h reaction and system appearance after solvent addition. Non-significant influences on reaction rates were observed although the reaction system turned clear after solvent addition in most cases. Slight increase of conversion rates was observed with 2-methoxyethanol and with the branched alcohols *iso*-propanol and *tert*-butanol whereas lower rates were detected with linear alcohols like 2-butanol and 1-propanol. *tert*-Butanol and 2-butanol have a

**Table II.** Enzyme activities for di-RL conversion and system appearance in dependence of co-solvent.

| Co-solvent | Addition (%) | Spec. activity (U mg$^{-1}$) | mono-RL mole fraction after 4 h | System appearance[a] |
|---|---|---|---|---|
| — | — | 2.37 | 0.90 | Turbid |
| — | — | 2.33 | 0.89 | Turbid |
| 2-Methoxyethanol | 5 | 2.35 | 0.91 | Clear |
| 2-Methoxyethanol | 10 | 2.47 | 0.93 | Clear |
| 1-Propanol | 5 | 2.00 | 0.86 | Clear |
| *iso*-Propanol | 5 | 2.35 | 0.89 | Turbid |
| *iso*-Propanol | 10 | 2.52 | 0.94 | Clear |
| 2-Butanol | 5 | 2.36 | 0.77 | Turbid |
| *tert*-Butanol | 5 | 2.41 | 0.89 | Turbid |
| *tert*-Butanol | 10 | 2.77 | 0.89 | Clear |
| DMSO | 5 | 1.92 | 0.86 | Clear |
| DMF | 5 | 1.44 | 0.79 | Clear |
| Dioxane | 5 | 2.30 | 0.90 | Clear |
| Dioxane | 10 | 2.38 | 0.93 | Clear |
| Butanone | 5 | 1.97 | 0.81 | Turbid |
| Acetonitrile | 5 | 1.95 | 0.87 | Clear |

[a]Solvent added to di-RL emulsion in sodium acetate buffer 0.1 M pH 4.5 and heated at 50°C.

damaging effect on enzyme activity with time as observed when comparing initial rates and conversion after 4 h. A minor increase on reaction rate was also reached with dioxane while all other non-alcohol-type solvents caused a decrease. Figure 3 shows specific activities in dependence of percentage of ethanol added to the reaction system. PnpR-activity decreased abruptly with ethanol addition evidencing its damaging effect. On the other hand, di-RL-activity decreased much less with solvent addition. To check whether ethanol penetrates in the RL micelle-phase and thus does not affect enzyme activity, ethanol effect on pnpR specific activities was assayed in the presence of di-RL micelle-phase. No protective effect of activity was detected suggesting that ethanol remains in the aqueous phase. Following conversions were conducted without co-solvent addition.

### Diffusion Effects

Figure 4A shows a linear correlation of initial reaction rate and enzyme concentration up to 0.26 $g\,L^{-1}$ Naringinase concentration when assaying bioconversion of 10 mM di-RL emulsions. This indicates enzymatically rate-controlled conditions as can be deduced from Equations (2) and (3). Since diffusion rate depends on the specific interfacial area *a* which increases with di-RL concentration, this linear correlation suggest that up to a mass ratio of enzyme to di-RL of 4%, rate limitation due to diffusion is negligible. Figure 4B shows specific activities in dependence of initial total di-RL concentration. The concentration of aqueous di-RL concentration in equilibrium $C_{\mathrm{di-RL}}^{\mathrm{aq,eq}}$ for every total di-RL concentration $C_{\mathrm{di-RL}}^{\mathrm{T}}$ is also plotted. Reaction rates and aqueous di-RL concentration remained constant throughout the concentration range 0.1–10 mM. This clearly shows that reaction rates are dependent on the aqueous, however, not on the total di-RL concentration. This is also in accordance with Equation (2). Since reaction rates were independent of the total RL concentration, and therefore of the specific interfacial area, this is an evidence of enzymatic reaction rate control. Correspondingly; the enzymatic reaction rate is much lower as compared to the diffusion rate of RL. Therefore, the aqueous bulk di-RL concentration $C_{\mathrm{di-RL}}^{\mathrm{aq}}$ equals the value in equilibrium; $C_{\mathrm{di-RL}}^{\mathrm{aq,eq}}$.

### Determination of Relevant Parameters

To compare theoretical predictions with experimental reaction courses, the parameters $cmc_{\mathrm{di\text{-}RL}}$, $A_s$, $K_m$, and $K_I$ of Equations (2)–(4) need to be known. If possible these parameters should be determined independently. The aqueous di-RL concentrations plotted in Figure 4B were determined as the aqueous concentration resulted after complete sedimentation of the micelle-phase. The average value obtained was $0.046 \pm 0.001$ mM (at pH 4.5, 60°C). Within experimental error, this value was slightly higher, but

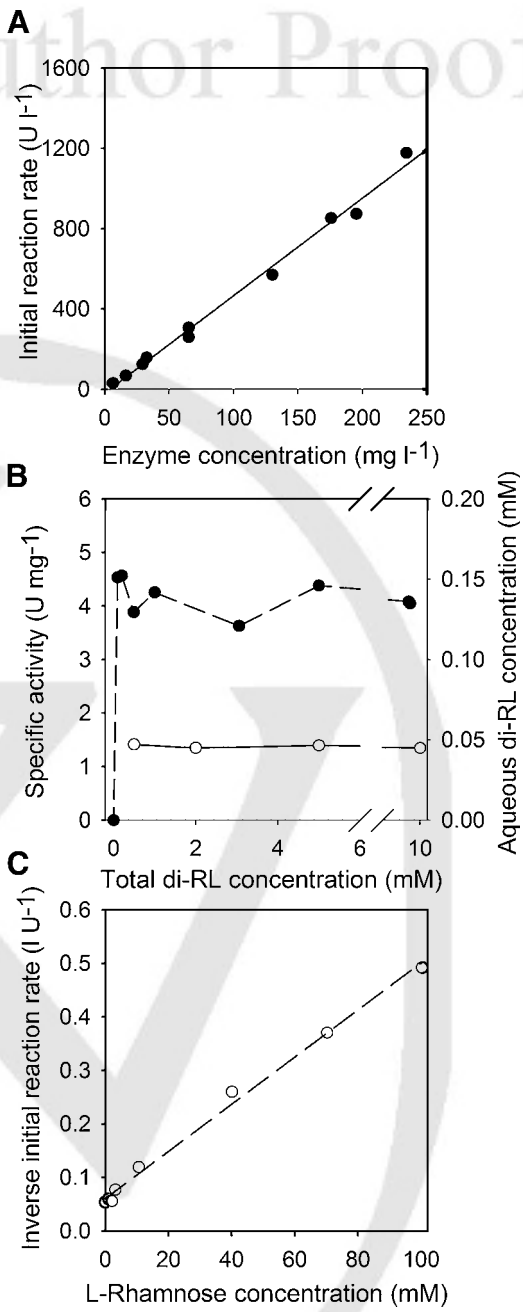


**Figure 4.** Effect on enzyme, di-RL and L-rhamnose concentration on initial reaction rates at 60°C and pH 4.5. **A**: Influence of enzyme concentration. **B**: Aqueous di-RL concentration (○) and influence of initial di-RL concentration (●). **C**: Dixon plot showing L-rhamnose inhibition. Dashed line: linear fitting for determination of inhibition coefficient.

of the same order of magnitude than the *cmc* of di-RL measured under the same conditions of pH and temperature (0.03 mM). This value was therefore applied as the concentration of free monomers. The rate constant ($A_s/K_m$) was determined taking the slope of the linear regression of Figure 4A (i.e., Eq. 2 under enzymatically

control at zero reaction time). A value of $0.104 \pm 0.004$ L min$^{-1}$ mg$^{-1}$ was therewith obtained. The absolute values $A_s$ and $K_m$ could not be evaluated. However, in Equations (2) and (3), respectively, only the quotient is required, provided that $K_m \gg C^{aq}_{di-RL}$. Figure 4C shows the influence of L-rhamnose concentration on the inverse initial reaction rate. L-Rhamnose decreased conversion rates probably due to competitive inhibition since this inhibition type was already observed with other substrates (Romero et al., 1985). A regression quality $R^2$ of 0.9948 was obtained when experimental points were fitted to the Dixon transformation of Equation (2) and an inhibition constant $K_{I,\ di\text{-}RL}$ of $9.7 \pm 1.1$ mM was therewith calculated. The kind of inhibition was not elucidated due to the difficulty to produce di-RL emulsions with varying di-RL aqueous concentrations.

Figure 5 shows mono-RL and di-RL aqueous concentration in dependency of mono-RL total mole fraction of 10 mM emulsions. An agreement between measured concentrations and the linear dependency assuming ideal surfactant mixing (Eq. 4) was observed. This is an expected correlation since mixtures of two non-ionic surfactants often behave ideally (Holland and Rubingh, 1992). By fitting the relation of aqueous to total mole fractions of mono-RL under ideal mixing to the experimental values of Figure 5 and taking $cmc_{di\text{-}RL}$ equals to 0.046 mM, the $cmc_{mono\text{-}RL}$ was estimated to be $0.033 \pm 0.002$ mM. Mixed emulsions of mono-RL and di-RL were apparently much more stable than pure mono-RL or di-RL emulsions and the micelle-phase could only be fully separated after 1 h centrifugation at 75,600*g*. The reason for this remains unclear.

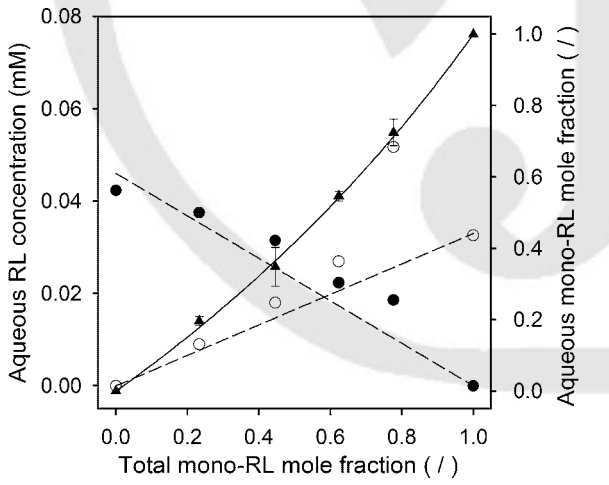


**Figure 5.** Effect of emulsion composition on the aqueous RL concentration: di-RL aqueous concentration (●), mono-RL aqueous concentration (○), and mono-RL aqueous mole fraction (▲). Dashed and full line: predictions considering ideal surfactant mixing.

## Comparison of Modeled and Experimental Reaction Courses

Equation (1) simplifies considerably when the rate limiting step is the enzymatic conversion. After rearrangement and replacing $C_{Rha}$ and $C^{aq}_{di-RL}$ as functions of $X^T_{di-RL}$, the following expression results:

$$\frac{dX^T_{mono-RL}}{dt} = \frac{(A_s/K_E)\rho_E\, cmc_{di-RL}\, X^T_{di-RL}}{C^T_{RL}\left[1 + \dfrac{CE(X^T_{di-RL,0} - X^T_{di-RL})}{K_{I,di-RL}}\right]} \frac{V_{aq}}{V_T} \quad (6)$$

Figure 6A–D shows experimental mono-RL mole fraction-time courses and its predictions by Equation (6). Experimental courses are well predicted within the whole bioconversion time. Moreover, results obtained by Equation (6) fits very well to experimental data considering variations in enzyme, di-RL and mono-RL concentrations. The goodness of fit between experimental and predicted is as high as 0.99 and parameter optimizations by fitting procedures were not required. Figure 6D shows bioconversion predictions of emulsions of higher RL concentrations. In this case, experimental observations were lower than predicted. Two reasons may account for this disagreement. First, at higher RL concentrations the volume of the micelle-phase cannot be neglected and the ratio of aqueous to total volume of Equation (6) can no longer be assumed to be unity. Since the micelle-phase consists of di-RL, mono-RL and water and assuming that most RL molecules are located in the micelle-phase the following relation can be derived:

$$\frac{V_{aq}}{V_T} = 1 - C^T_{RL}\frac{W_m}{X^m_{RL}} \quad (7)$$

In Equation (7), $W_m$ is the molar volume of the micelle-phase and $X^m_{RL}$ the mole fraction of RL in the micelle-phase. After settling down the micelle-phase in a 100 mM di-RL emulsion, it was established that 1.6 g water per g di-RL is found in the micelle-phase. Therefore, $X^m_{RL}$ equals 0.017. The micelle-phase molar volume was estimated from the molar volume of water and of RL considering its mole fractions as the weighting factor. Molar volumes of di-RL and mono-RL were estimated to be 0.5279 and 0.441 L mol$^{-1}$ at 20°C, respectively (SciFinder Scholar Database, American Chemical Society, WA). Taking the mean of the above molar volumes, a value of 0.026 L mol$^{-1}$ was therewith obtained for the micelle-phase. From a settled 100 mM di-RL emulsion at 60°C the ratio $V_{aq}$ to $V_T$ was found to be 0.82 and $W_m$ can be calculated from Equation (7) as 0.029 L mol$^{-1}$. Thus, estimated and experimentally observed mole volumes were in accordance. Taken into account Equation (7) for modeling with $W_m$ equals to 0.029 L mol$^{-1}$, predictions were re-calculated and are shown in Figure 6D as dashed lines. An enhanced prediction to experimental was achieved mainly for 91 mM emulsion.

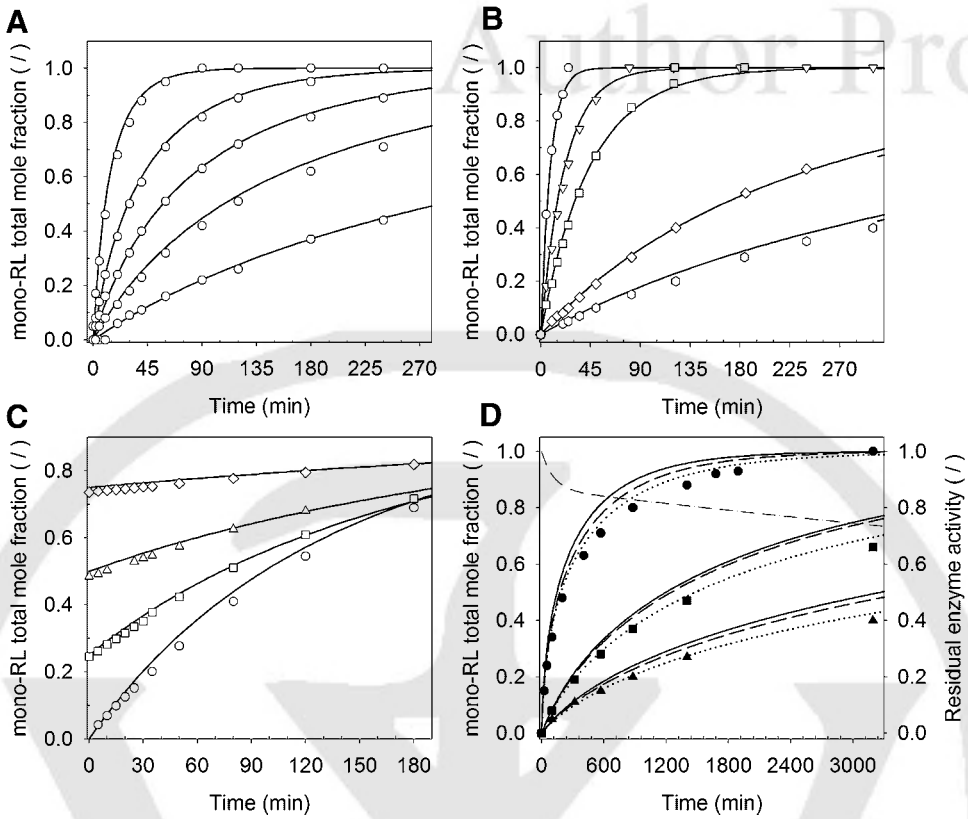


**Figure 6.** Prediction by Equation (6) (full lines) and experimental values (points) of mono-RL mole fraction during different set of biotransformations at pH 4.5 and 60°C. **A**: 195.2, 65.0, 32.5, 16.2, and 6.5 mg $L^{-1}$ enzyme concentration, respectively. **B**: 10, 5, 1, 0.5, and 0.2 mM di-RL, respectively. **C**: 0, 0.25, 0.5, and 0.75 initial mono-RL mole fraction, respectively. **D**: 91 mM di-RL (●), 47 mM di-RL (▲), 24 mM di-RL (■); dashed line: Equation (6) with volume ratio correction; dotted line: Equation (6) with volume ratio correction and enzyme deactivation; dash-dotted line: residual enzyme activity.

The second reason for disagreement may have been enzyme deactivation during the longer conversion time. Considering a series-type thermal deactivation model of Naringinase, a correction factor was integrated in Equation (6). Thus, the reaction rate $r$ was multiplied by $a(t)$, the residual enzyme activity, which for Naringinase at reaction conditions (60°C, pH 4.5 and absence of rhamnolipid) equals (Magario et al., 2008a):

$$a(t) = 0.14\,e^{-k_1 t} + 0.86\,e^{-k_2 t} \qquad (8)$$

The kinetic constant $k_1$ and $k_2$ are equal to $8.67 \times 10^{-3}$ and $5 \times 10^{-5}$ $min^{-1}$, respectively (Magario et al., 2008a). The dotted lines of Figure 6D correspond to the prediction of Equation (6) multiplied by the factor $a(t)$ and considering volume ratio correction. The residual enzyme activity is also plotted as the dash-dotted line. A better agreement to experimental values ($R^2 = 0.99$) was therewith obtained. Thus, enzyme deactivation as well as volume ratio correction should be taken into account when biotransformations at high rhamnolipid concentrated emulsions are assayed during a larger reaction time.

### Consecutive mono-RL Conversion

Naringinase is known to convert mono-RL into L-rhamnose and di-HDA however at much lower rates as compared to di-RL conversion (Meiwes et al., 1997; Trummler et al., 2003). Figure 7A shows the reaction course of mono-RL conversion by Naringinase. Equation (6) can easily be modified for mono-RL-conversion provided ideal mixing of the surfactants mono-RL and di-HDA. By fitting Equation (6) to the experimental points of Figure 7A the parameters $A_s/K_m$ and $K_I$ for mono-RL as substrate were deduced. Since substrate and product in this case are mono-RL and di-HDA, respectively, the mole fraction of Equation (6) were replaced accordingly for modeling the second reaction. A $A_s/K_m$ value of 0.0019 L $min^{-1}$ $mg^{-1}$ and a $K_I$ value of 1.2 mM were hence obtained with a goodness of fit of 0.99 taking into account enzyme deactivation during reaction according to Equation (8). The mono-RL mole fraction can be

modeled considering the consecutive reaction by subtracting to the right term of Equation (6) the consumption term due to mono-RL-bioconversion:

$$\frac{dX^{T}_{mono-RL}}{dt} = \frac{(A_s/K_m)_{di-RL}\rho_E\, cmc_{di-RL}\, X^{T}_{di-RL}}{C^{T}_{RL}\left[1+\dfrac{C^{T}_{RL}(X^{T}_{di-RL,0}-X^{T}_{di-RL})}{K_{I,di-RL}}\right]} - \frac{(A_s/K_m)_{mono-RL}\rho_E\, cmc_{mono-RL}\, X^{T}_{mono-RL}}{C^{T}_{RL}\left[1+\dfrac{C^{T}_{RL}(1+X^{T}_{di-RL,0}-2X^{T}_{di-RL}-X^{T}_{mono-RL})}{K_{I,mono-RL}}\right]} \quad (9)$$

Figure 7B shows the reaction course starting from a di-RL mole fraction equals to 1. di-RL is nearly totally converted before mono-RL bioconversion becomes detectable and this could be properly be predicted by Equation (9). Due to product inhibition by the accumulated L-rhamnose from the former conversion, the higher the initial di-RL concentration, the lower the subsequent mono-RL conversion rate should be. This effect could be utilized as an additional control parameter for avoiding the subsequent mono-RL conversion. Table III gives an overview of all parameter values used for prediction equations.

## Discussion

A number of publications deal with modeling of enzymatic reactions with mixed micellar substrates assuming enzyme adsorption in the interface. Although kinetic courses could be predicted by assuming reaction in the aqueous phase, enzyme adsorption to the micelles with consequent interfacial hydrolysis can also occur. Straathof (2003) postulated that the location of the reaction is difficult to determine from measurement of changes in substrate concentration alone. In the case of interfacial reaction the first step is the fixation of a water-soluble enzyme to a lipid/water interface followed by a 2D Michaelis–Menten catalytic step (Panaiotov et al., 1997). However, the enzymes involved there were lipases and phospholipases, some of them showing interfacial activation, and whose natural substrates are insoluble and located into aggregates (Panaiotov et al., 1997; Rubingh, 1996). On the other hand, Naringinase used in this study is not expected to adsorb to an interface for substrate attack and no observations were made that may indicate interfacial activation. To our knowledge there are no glycosidases other than cellulases showing interfacial activity. The terms ''interfacial activation'' or ''interfacial catalysis'' seems only to be applicable for lipases and phospholipases (Straathof, 2003). Chopineau et al. (1998) observed from kinetic studies that β-D-glucosidase did not accept micelles as substrates but only the monomeric form of octyl-β-D-glucopyranoside. Furthermore, there are some observations supporting the idea of aqueous phase reaction: (1) in the case of bioconversion of micellar substrates with lipases a Michaelis–Menten behavior between reaction rate and total bulk concentration was often observed (Deems et al., 1975; Redondo et al., 1995; Rubingh and Bauer, 1992). In the present case the reaction rate was independent of the total bulk RL concentration indicating that aqueous reaction may apply; (2) in co-incubation experiments, Naringinase was able to cleave pnpR in the presence of the RL micelle-phase in a similar rate that without RL. This may indicate that most enzyme molecules remained in the aqueous phase; (3) recent results regarding kinetic data of di-RL conversion with immobilized Naringinase on porous supports (Magario et al., 2008b), could be interpreted considering

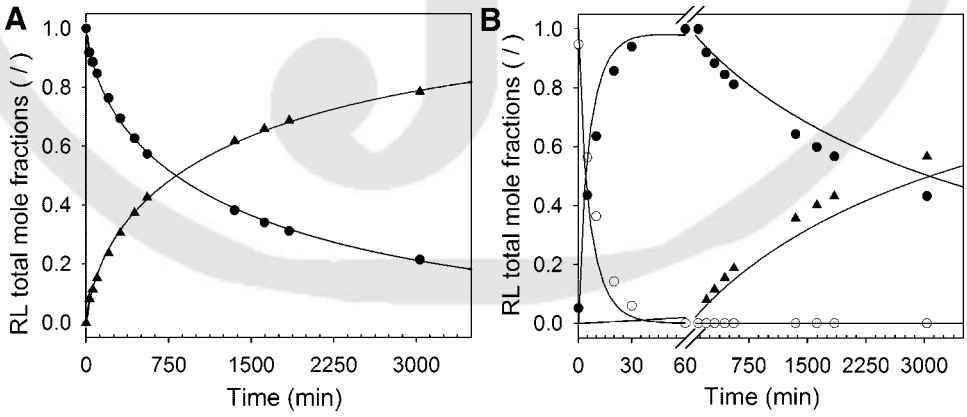


**Figure 7.** mono-RL bioconversion. **A**: Bioconversion starting from mono-RL. **B**: Bioconversion starting from di-RL. Experimental mole fractions of di-RL (○), mono-RL (●), and di-HDA (▲). Lines: Prediction by the opposite of Equation (6) for di-RL mole fraction, Equation (9) for mono-RL mole fraction and the opposite of the second term of the right side of Equation (9) for di-HDA mole fractions.

**Table III.** Value of the equations parameters.

| | *cmc* (mM) | $A_s/K_m$ ($L\,min^{-1}\,mg^{-1}$) | $K_I$ (mM) | $W_m$ ($L\,mol^{-1}$) |
|---|---|---|---|---|
| di-RL | $0.046 \pm 0.001$[a] | $0.104 \pm 0.004$[a] | $9.7 \pm 1.1$[a] | 0.029[a] |
| mono-RL | $0.033 \pm 0.002$[a] | $0.0019 \pm 0.00026$[b] | $1.2 \pm 0.3$[b] | |

[a]Experimental determination.
[b]Determined by fitting equation 6 to the experimental points of Figure 7A. Initial parameters values were set at 0.001 $L\,min^{-1}\,mg^{-1}$ for $(A_s/K_m)_{mono\text{-}RL}$ and at 9.7 mM for $K_{I,mono\text{-}RL}$.

high intern diffusion-limited reaction rate due to the very low aqueous di-RL concentration.

Low-molecular alcohols are known to increase the *cmc* of non-ionic surfactants due to a weakening of its hydrophobic bonding. Higher alcohols cause however a *cmc* decrease as a consequence of its penetration into the micelles (Attwood and Florence, 1983). Assuming aqueous reaction applies, variation in *cmc* of di-RL should strongly affect reaction rates. However, when trying to increase *cmc* values by addition of co-solvents or varying bulk pH increasing reaction rates were not observed and an overall little effect was detected. Changes in the reaction rates are presumably the result of two coupled phenomena, namely increased reaction rates due to increased monomer availability (*cmc*) and decreased enzyme activity due to condition variations. This explanation may be supported by the observation that decreasing reaction rate due to alcohol addition or bulk pH are more pronounced for other substrates like pnpR (Romero et al., 1985).

Romero et al. (1985) determined kinetic data with pnpR and Naringin at optimal conditions (pH 3.5 and 57°C): $A_s$ values of 10.7 and 150 $U\,mg^{-1}$; $K_m$ values of 1.52 and 7.0 mM. This results in $A_s/K_m$ values of 0.007 and 0.021 $L\,min^{-1}\,mg^{-1}$ for pnpR and Naringin, respectively. This indicates that the substrate specificity of Naringinase is in the sequence: di-RL > Naringin > pnpR > mono-RL (see Table III) and following conclusions may be extracted: (1) The $1 \rightarrow 2$ Rha-Rha linkage of di-RL can be hydrolyzed more efficiently than the $1 \rightarrow 2$ Rha-glucose linkage of Naringin; (2) L-rhamnose is cleaved from mono-RL much slower than from pnpR probably due to steric hindrance of the larger aglycone portion of mono-RL. Moreover, the larger specificity towards substrates as di-RL or Naringin compared with pnpR and mono-RL is in agreement with the findings of Michon et al. (1989), who established that Naringinase can hydrolyze more rapidly L-rhamnose from glycosidic linkage rather than from an aglycone linkage.

## Conclusion

From this study it can be concluded that Naringinase from *P. decumbens* is an appropriate catalyst for the bioconversion of di-RL into mono-RL and L-rhamnose.

Kinetic data of the hydrolysis of an aggregating substrate, di-RL by Naringinase was properly modeled. The apparent non-Michaelis–Menten behavior was interpreted by assuming an enzymatically rate-controlled reaction in the aqueous phase. Moreover, the well predicted effects to changes in the initial mono-RL mole fraction suggest that the strong influence of mono-RL on reaction rates is exclusively due to ideal surfactant mixing, and further effects like product inhibition are negligible. This approach may also be applied when RL other than di-RL and mono-RL are present in the reaction system. Moreover, the findings of this study may beneficially be adapted for any bioconversions involving aggregate-forming substrate and/or product being catalyzed by hydrophilic enzymes.

## Nomenclature

| | |
|---|---|
| $a$ | interfacial area relative to the aqueous phase ($m^{-1}$) |
| $A_s$ | maximal specific enzyme activity ($mmol\,min^{-1}\,mg^{-1}$) |
| $a(t)$ | residual enzyme activity |
| $C$ | substrate or product concentration (mM) |
| *cmc* | critical micelle concentration |
| di-HDA | 3-(3-hydroxydecyloxy)decanoic acid |
| $k_1$ and $k_2$ | enzyme deactivation constants ($min^{-1}$) |
| $K_I$ | rhamnose inhibition constant (mM) |
| $k_{L,di\text{-}RL}$ | di-RL mass transfer coefficient ($m\,min^{-1}$) |
| $K_m$ | Michaelis–Menten constant (mM) |
| pnpR | p-nitrophenyl-L-α-rhamnoside |
| $r$ | enzymatic reaction rate ($mmol\,L^{-1}\,min^{-1}$) |
| mono-RL | mono-rhamnolipid or rhamnolipid 1 |
| di-RL | di-rhamnolipid or rhamnolipid 3 |
| Rha | L-rhamnose |
| RL | rhamnolipids |
| $V$ | volume (L) |
| $W_m$ | molar volume of the micelle-phase ($L\,mol^{-1}$) |
| $X$ | mole fraction |
| $\phi_{di\text{-}RL}$ | di-RL mass transfer rate; $\phi_{di-RL} = k_{L,di-RL}a(C^{aq,eq}_{di-RL} - C^{aq}_{di-RL})$ ($mmol\,min^{-1}\,L^{-1}$) |
| $\rho_E$ | enzyme mass concentration ($mg\,L^{-1}$) |

*Sub- and Super-Indices*

| | |
|---|---|
| aq | aqueous phase |
| eq | in equilibrium |
| m | micelle-phase |
| T | sum of aqueous and micelle phases |

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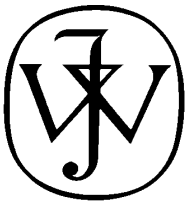

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