# Enantioselective Transesterification by *Candida antarctica* Lipase B Immobilized on Fumed Silica

Short Running Title

*Candida antarctica* Lipase B Immobilized on Fumed Silica

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Please cite this article as: Kramer, M., Cruz, J.C., Pfromm, P.H., Rezac, M.E., Czermak, P., Enantioselective Transesterification by Candida antarctica Lipase B Immobilized on Fumed Silica, *Journal of Biotechnology* (2010), doi:10.1016/j.jbiotec.2010.07.018

**Keywords:**
*Candida antarctica* Lipase B; hexane; enzyme immobilization; fumed silica; enantioselective transesterification; enzyme stability

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## Abstract

Enzymatic catalysis to produce molecules such as perfumes, flavors, and fragrances has the advantage of allowing the products to be labeled "natural" for marketing in the U.S., in addition to the exquisite selectivity and stereoselectivity of enzymes that can be an advantage over chemical catalysis. Enzymatic catalysis in organic solvents is attractive if solubility issues of reactants or products, or thermodynamic issues (water as a product in esterification) complicate or prevent aqueous enzymatic catalysis. Immobilization of the enzyme on a solid support can address the generally poor solubility of enzymes in most solvents.

We have recently reported on a novel immobilization method for *Candida antarctica* Lipase B on fumed silica to improve the enzymatic activity in hexane. This research is extended here to study the enantioselective transesterification of (RS)-1-phenylethanol with vinyl acetate. The maximum catalytic activity for this preparation exceeded the activity (on an equal enzyme amount basis) of the commercial Novozyme 435® significantly. The steady-state conversion for (R)-1-phenylethanol was about 75% as confirmed via forward and reverse reaction. The catalytic activity steeply increases with increasing nominal surface coverage of the support until a maximum is reached at a nominal surface coverage of 230%. We hypothesize that the physical state of the enzyme molecules at a low surface coverage is dominated in this case by detrimental strong enzyme-substrate interactions. Enzyme-enzyme interactions may stabilize the active form of the enzyme as surface coverage increases while diffusion limitations reduce the apparent catalytic performance again at multi-layer coverage. The temperature-, solvent-, and long-term stability for CALB/fumed silica preparations showed that these preparations can tolerate temperatures up to 70°C, continuous exposure to solvents, and long term storage.

# Introduction

Non-aqueous enzymology is an attractive option for the synthesis of various molecules in non-aqueous media (Hudson et al., 2005; Klibanov, 2001). Promising experiments for polymer synthesis (Gross and Kalra, 2002; Gross et al., 2001), anticancer and antiviral drugs (Gotor, 2002), aromas and fragrances (Barahona et al., 2006; Bartling et al., 2001), and surfactants (Bruno et al., 1995) have been reported.

The performance of biocatalysis in organic solvents significantly extends the applications of biocatalysts. This is mainly because water is a poor solvent for many organic compounds of commercial interest (Ghanem, 2007). Furthermore, undesirable side reactions such as hydrolysis, racemization, polymerization and decomposition are largely repressed in solvents when compared to aqueous solution (Ghanem, 2007; Klibanov, 2001).

Nevertheless, the catalytic activity of enzymes in organic solvents tends to be much lower than in aqueous environments (Ghanem, 2007; Klibanov, 2001).

To overcome this issue, different approaches have been developed. Immobilization of enzymes on porous and non-porous solid supports has been intensively explored (Long et al., 2007; Persson et al., 2002). The preferred matrices for immobilization include macroporous polypropylene particles (Bosley and Peilow, 1997), hydrophilic silicon wafers (van der Veen et al., 2007), microemulsions and organogels (Zoumpanioti et al., 2008). Additional efforts include improving compatibility with the solvents by chemical modification of the enzymes' surface (Sheldon et al., 2005), protein engineering (Hudson et al., 2005), and co-lyophilization of the enzyme with various adjuvants, such as cyclodextrin (Ghanem, 2003; Mine et al., 2003), inorganic salts (Lindsay et al., 2002, 2004), and crown ethers (Mine et al., 2003; Santos et al., 2001; Secundo et al., 2007).

The immobilization on organic and modified inorganic nano-structured supports is considered now as an attractive option for immobilization. A variety of these materials is available including epoxy-activated nanobeads, zirconia nanoparticles, and fumed silica. The main advantage is the large specific surface area provided by such materials (Chen et al., 2008a; Chen et al., 2008b; Cruz et al., 2009). We have recently reported on a new immobilization technique for the activation of two different enzymes on commercial fumed silica (FS): *Candida antarctica* Lipase B (CALB) (Cruz et al., 2009) and *subtilisin Carlsberg* (Cruz et al., 2009; Pfromm et al., 2007; Wurges et al., 2005). The enzyme is co-lyophilized from the aqueous phase with fumed silica. The significant activation of the enzyme reached or in some cases even exceeded the best activities reported for salt activation while the process is somewhat simplified (Cruz et al., 2009; Pfromm et al., 2007). The details can be found elsewhere (Cruz et al., 2009; Pfromm et al., 2007; Wurges et al., 2005).

The main driving forces for protein adsorption on solids are thought to be of hydrophobic and electrostatic nature. These interactions are essentially driven by the net charge difference between the protein and the surface (Cruz et al., 2009; Koops et al., 1999). An important issue associated with the adsorptive immobilization of enzymes is that conformational changes are sometimes observed (Bosley and Peilow, 1997; Koops et al., 1999; Unsworth et al., 2007; van der Veen et al., 2007). These conformational changes may modify the native enzyme structure and promote fluctuations in activity at low enzyme loadings (in this work, referred as low nominal surface coverage). Thus, a large excess of surface area maximizes the interactions of the lipase with the surface, which can result in the above described structural changes and in reduced activity (Bosley and Peilow, 1997; Koops et al., 1999).

To retain more lipase molecules in the active conformations after adsorption, one may consider mechanisms to suppress the tendency of the enzyme to deform when sufficient surface area is provided. This might be achieved by increasing the presence of neighboring molecules at increased surface coverages (Bosley and Peilow, 1997; Koops et al., 1999; van der Veen et al., 2007). Mass-transfer limitations may start to be significant at multi-layer coverages thereby, potentially leading to reduced apparent activity. The overall result is low apparent activity at low surface coverage, maximum apparent activity at an intermediate coverage, and again low activity at high or multi-layer coverages (Bosley and Peilow, 1997; Koops et al., 1999).

This work reports on the performance of *Candida antarctica* Lipase B immobilized on fumed silica in an enantioselective transesterification reaction in hexane. Our findings reproduce the maximum of catalytic activity at an intermediate surface loading, reported previously for a non-stereoselective reaction (Cruz et al., 2009). We also investigated the steady-state conversion, the thermal stability, the solvent stability, and the long-term stability at 4°C for preparations with various surface coverages.

# Material and Methods

**Enzymes**

Crude CALB (E.C. 3.1.1.3; lyophilized; specific activity of 30 U/mg solid) was purchased from Biocatalytics, Inc. (Pasadena, CA), stored at 4°C, and used as-received. Commercial Novozyme 435® was purchased from Sigma, stored at 4°C, and used as-received. Novozyme 435® is a preparation of the lipase B from *Candida antarctica* adsorbed on macroporous acrylic particles (0.3-0.9 mm diameter) reported to have about 7000 PLU/g (Propyl Laurate Units per gram preparation).

**Chemicals and materials**

Monobasic potassium phosphate (>99.9%), potassium hydroxide, hexane (98.5%, Acros Organics), isopropanol (99.9%, Fisher, Sigma-Aldrich), vinyl acetate (99%, Alfa Aesar), acetaldehyde (99.5%, Acros Organics, stored at 4°C) were purchased from Fisher Scientific (Pittsburg, PA).

The chemicals for calibration, enzymatic reactions, immobilization, and Karl Fischer titration, α-methylbenzyl acetate (98+%, SAFC sampling solutions), (S)-1-phenylethanol (97%, Sigma-Aldrich), (RS)-1-phenylethanol (98%, Sigma-Aldrich), HYDRANAL®-Coulomat AK anolyte (Fluka), HYDRANAL®-Coulomat CG-K catholyte (Fluka), and Fumed Silica (Product number 381276, 99.8%, specific surface area 255$m^2$/g, primary particle diameter ≅ 7-50 nm, as reported by the manufacturer) were purchased from Sigma-Aldrich (St. Louis, MO). A chiral High Performance Liquid Chromatography (HPLC) column (Chiralcel OD-H, 0.46 cm inner diameter, 25 cm length; Daicel Chemical Industries, Tokyo, Japan) and a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) were used (Pumps LCD-10ATvp liquid chromatography, degasser DGU-14A, auto injector SIL-10ADvp, system controller SCL-10Avp, column oven CTO-10Avp, diode array detector SPD-M10Avp, Shimadzu Chromatography Laboratory Automated Software System Version 7).

A 20 µL-200 µL and a 100 µL-1000 µL Finnpipette (Fisher Scientific) were used for pipetting. 1.5 mL glass vials and caps with pre-assembled septa for HPLC sampling were from Sun Sri. For storage the septum caps were replaced with solid caps (Fisherbrand, Fisher Scientific).

To carry out the enzymatic reactions a PsyCro Term Controlled Environment Incubator Shaker (New Brunswick Scientific) was used. Reactions were performed in 12 mL glass vials

with open top caps and septa from National Scientific. Solvent and temperature stability experiments were performed using solid caps (Kimble). 4 oz glass jars with plastic screw caps were used for conversion experiments (Wheaton). A 1 mL gastight syringe with Luer Lock head and 3 inch needles (Hamilton, Reno, NV) was used for sampling. Samples were filtered with 0.2 μm PTFE syringe filters (Whatman Inc.).

**Enzyme surface coverage of the support**

The surface coverage of the solid support (fumed silica) by the immobilized enzyme is tracked here by calculating a nominal surface coverage based on the projected surface area of a CALB molecule of 28.27nm$^2$ determined via x-ray crystallography resulting in a spherical CALB molecule of 6nm diameter (Uppenberg, 1994). The surface coverage is determined according to

$$\%SC = 100\frac{nA_E}{A_S} \qquad \textbf{Equation 1}$$

where %SC is the nominal surface coverage in %, n is the number of moles of enzyme, $A_E$ is the projected surface area of an enzyme molecule in m$^2$, and $A_S$ is the surface area of the fumed silica support in m$^2$ as reported by the manufacturer. Both the spherical shape of the enzyme and the surface curvature of the support cause *actual* 100% monolayer surface coverage to likely occur below 100%SC *nominal* surface coverage. This is exacerbated if the enzyme molecules deform to maximize enzyme-support interactions. In summary, actual monolayer coverage is expected below a calculated nominal 100%SC. Multi layer coverage will lead to nominal surface coverages calculated above 100%SC.

**Enzyme immobilization**

Details for this process can be found elsewhere (Cruz et al., 2009; Wurges et al., 2005). In a glass vial crude CALB and aqueous buffer were mixed under vortexing for about 30 s. After

adding fumed silica the preparations were homogenized by vortexing followed by sonication for 10 min. The suspension was then stored at -20°C in a refrigerator until frozen. The frozen samples were lyophilized (48 h primary drying, 24 h secondary drying, VirTis model 10-MR-TR: Gardiner, NY).

Table 1 shows the amounts of aqueous buffer and fumed silica needed for the various % SC-preparations.

Our preparations and Novozyme 435® are compared here on an equal PLU basis. As reported elsewhere ((Chen et al., 2008a), active site titration in organic media) the fraction of active enzyme in the Novozyme 435® immobilizate is about 50.3%. The measured initial reaction rates for Novozyme 435® were corrected to compare with our preparations using the relationship

$$r_{0plotted} = r_{0\ measured} * 0.503 \qquad \textbf{Equation 2}$$

where $r_0$ is the initial reaction rates in μmol/s.

**Mobile phase**

The mobile phase used for HPLC was a hexane/isopropanol mixture (9:1, v/v). The stock solution was composed of 3600 mL of hexane and 400 mL of isopropanol and stored in amber glass vessels sealed against the open atmosphere.

**Analytical Methods for Reactions in Hexane**

Substrates ((RS)-1-phenylethanol, vinyl acetate) and products ((R)-1-phenylethyl acetate, (S)-1-phenylethanol) were tracked in the reaction mixture by HPLC. The retention times were 6.5, 7.2, 10.3, and 11.1 min for vinyl acetate, (R)-1-phenylethyl acetate, (R)-1-phenylethanol, and (S)-1-phenylethanol; respectively. Kinetic parameters are based on the appearance of (R)-1-

phenylethyl acetate. The conversion is defined in terms of a fractional conversion c as (Chen et al., 1982):

$$c = 1 - \frac{[R]+[S]}{[R]_0 + [S]_0} \qquad \textbf{Equation 3}$$

where [R] is moles of (R)-1-phenylethanol from analysis, [S] is moles of (S)-1-phenylethanol from analysis, $[R]_0$ is the initial amount of (R)-1-phenylethanol in moles, and $[S]_0$ is the initial amount of (S)-1-phenylethanol in moles

**Initial Reaction Rates**

The enzyme preparation was weighed into 12 mL glass vials. Table 1 shows the needed weights for the different preparations. 6 mL hexane were then added. As substrates 42.4 mg vinyl acetate (82 mM) and 60 mg (RS)-1-phenylethanol (82 mM) were finally added and the time of adding the reactants was defined as time zero.

The vials were then placed in the incubator (30°C, 280 rpm). 400 µL samples are taken every 20 min with 1 mL gastight syringes and are afterwards directly filtered with syringe filters pre-purged with hexane. 200 µL are pipetted into 1.5 mL HPLC vials with 1100 µL mobile phase. These HPLC vials are refrigerated until used.

**Thermal Stability**

Temperature stability experiments were carried out with enzyme preparations of 230 %SC, 100 %SC, 12 %SC, and 2 %SC nominal surface coverage and for Novozyme 435® by measuring the initial reaction rates at 45°C, 60°C, and 70°C.

**Steady-State Conversion**

To determine the steady-state conversion forward reactions with a reaction mixture volume of 48 mL were performed. The amounts of preparations and substrates were properly scaled-up.

Preparations with a range of nominal surface coverages between 400 %SC and 4 %SC were tested. The reactions were tracked by measuring the (RS)-1-phenylethanol consumption. The reverse reaction with the 230%SC preparation was performed to check the accuracy of the steady-state condition and the experimental procedures.

**Hexane Storage Stability**

Preparations were suspended in 6 mL hexane and incubated (280 rpm, 30°C) for up to 4 days. The substrates were then added and initial reaction rate experiments were carried.

**Long-Term Stability for Storage at 4°C**

Initial reaction rates of enzyme preparations (17, 4, and 2 %SC) that had been stored dry for one year at 4°C in glass vials closed with screw caps were determined (see procedure above).

**Water content analysis**

The water content of our reaction mixture was measured by coulometric Karl Fischer titration (Denver Model 275 KF titration module, Model 270 controller, Denver Instruments) of about 1 mL samples taken with a gas tight syringe (5mL, Hamilton).

Due to the presence of the keto group (=C=O) in the vinyl acetate, it is likely that interference with standard Karl Fischer reagents occurs (Vantol et al., 1995). Karl Fischer reagents HYDRANAL®-Coulomat AK and HYDRANAL®-Coulomat CG-K (both Fluka purchased from Sigma-Aldrich) were therefore used. Titration of a HYDRANAL® Water Standard (Riedel-de Haën, 100mg water/g) in six independent titrations in the same titration module resulted in an average of 99.6 ppm $H_2O$ (standard deviation 4.4 ppm).

Syringes were carefully cleaned and always stored in a desiccator. The water concentration in our reaction mixture (6 mL hexane, 60µL (RS)-1-phenylethanol (82 mM), 45µL vinyl acetate

(82 mM), appropriate amount of CALB/FS preparation) was determined after filtering (0.2 μm syringe filters) the homogenized mixtures three times.

## Results and Discussion

We report the enantioselective catalytic activity of CALB in hexane immobilized on a non-porous inorganic support that consists of nano-scale spherical silica particles fused into necklace-like arrangements (fumed silica) (Iler, 1979). The simplicity of the procedure to produce the preparation, the low cost and availability of the fumed silica (commercially available both native and with surface modifications), and the proven ability to operate the preparation in a packed bed continuous reactor are attractive (Pfromm et al., 2007; Wurges et al., 2005).

The good match of the conversion (about 75%, **Fig. 1**) of the forward and reverse reactions for the transesterification of (R)-1-phenylethanol with vinyl acetate as acyl donor catalyzed by CALB immobilized on fumed silica in hexane indicates that our experimental and analytical procedures appear consistent. Comparison with the literature confirms this in hexane (Sriappareddy et al., 2007) and other organic solvents (Han et al., 2006; Kamori et al., 2002). The water content in our reaction mixtures was on average 33±5.5 ppm. This corresponds to a thermodynamic water activity $a_w$ of 0.38±0.06 assuming a saturation level of 89.4ppm of water in hexane at the reaction conditions (Kang et al., 2005).

The initial reaction rates (2 hrs) for different nominal surface coverages show a maximum at an intermediate nominal surface coverage of about 230%SC (**Fig. 2**). This indicates multi-layer coverage if the geometry of the enzyme molecule and the available fumed silica surface assumed in the calculation of %SC is correct. This is similar to previous observations (Bosley and Peilow, 1997; Cruz et al., 2009; Wurges et al., 2005) although the maximum occurs at different nominal surface coverages (discussion below). The best reaction rate doubles that of commercial

Novozyme 435® on an equal PLU basis. The different CALB immobilizates on fumed silica all showed the same high enantioselectivity; >99% (data not shown) as reported by others (Han et al. 2006).

Based on the literature and our results (Bosley and Peilow, 1997; Cao, 2005; Cruz et al., 2009; Janssen et al., 2002; Koops et al., 1999), we postulate here that three phenomena contribute to the observed catalytic activity maximum: 1.detrimental conformational changes of enzyme molecules upon adsorption on a solid (Bosley and Peilow, 1997; Koops et al., 1999), 2.beneficial interaction of adsorbed enzymes with neighboring enzyme molecules at increasingly "crowded" conditions, and 3. reactant and/or product diffusional limitations due to multi-layer deposition of the enzyme (Cao, 2005; Janssen et al., 2002; Koops et al., 1999). A more detailed mechanistic explanation of each situation is given below and schematically shown in **Fig. 3**.

**Fig. 2** can be interpreted in light of the three effects above. The possible detrimental structural changes of the enzyme after adsorption on the solid may impact the more sensitive stereoselective reaction more severely than the simple esterification of geraniol. More stabilization may therefore be needed to reach the maximum reactivity for the more complex stereoselective catalytic process. This corresponds to a higher nominal surface coverage needed to reach the maximum reactivity, pointing towards more protein/protein interactions. The decline in reactivity then follows when increasing multi layer coverage causes mass transfer limitations. This is schematically shown in **Fig. 3** where three regimes of surface loading are proposed:

1. Low nominal surface coverage where most enzyme molecules adsorb isolated from each other thereby promoting conformational changes (by deformation upon multipoint attachment to the surface). Few active enzyme molecules are responsible for catalysis, which explains the

considerable loss of activity per enzyme molecule in this regime. The presence of this conformation-controlled regime has been previously suggested by others (Cao, 2005).

2. High catalytic activity per enzyme molecule at intermediate surface coverage where enzymes are likely adsorbed on a previously formed enzyme monolayer and stabilized by enzyme-enzyme interactions.

3. A high nominal surface coverage regime with multiple layers where diffusional mass transfer barriers result in a low apparent catalytic activity per enzyme molecule. The existence of this mass transfer-controlled regime was also suggested previously (Cao, 2005).

**Fig. 4** shows the impact of temperature on the catalytic activity of our preparations. All enzyme preparations show Arrhenius-type activation from 30°C to 45°C. The 2 %SC preparation remains at the same albeit low level of activity for all temperatures. This can be interpreted to support the notion that the enzyme molecules in this preparation may have strong interactions with the support due to the large surface area provided. This enhanced contact is likely to lead to detrimental impact on the structure (resulting in low activity) but on the other hand helps to maintain the integrity of the still active molecules at higher temperatures (Koops et al., 1999). The 12 %SC preparation shows a similar progression, however, the denaturing effect of increased temperature is more pronounced. Thus, after reaching a maximum at 45°C the activity decays continuously.

Higher activity values for the 100 %SC and 230 %SC preparations were observed at all temperatures exceeding those of Novozyme 435®. Additionally, the 100 %SC shows a 2.5-fold increase in activity from 30°C to 60°C and a precipitous decrease from 60°C to 70°C. The activity of the 230 %SC preparation, however, increased 1.5-fold from 30°C to 45°C and remained at the same high level above 60°C indicating that the enzyme localization on the

surface largely prevents thermal damage and the subsequent break down of the catalytic machinery.

In summary, when superimposed; the three effects of denaturation by enzyme-solid interaction, stabilization by enzyme-enzyme interaction, and mass transfer limitation at multi layer coverage lead not only to a maximum in the activity as a function of coverage but to very stable conformations that can tolerate relatively high temperatures. This represents a tremendous advantage from the processing stand point as a number of processes of industrial importance are preferentially performed at temperatures above the ambient condition.

The temperature dependence of *R. miehei* lipase immobilized in lecithin based microemulsion-based organogels is superimposed in **Fig. 4** for comparison (Zoumpanioti et al., 2008). **Fig. 4** shows that these lipase preparations are behaving similarly as the temperature is raised from 30°C to 70°C. This confirms that our system exhibits a similar temperature dependency as those reported elsewhere (Zoumpanioti et al., 2008). This further supports that our preparations can activate to a level that is commonly obtained for the same enzyme immobilized on supports with different chemical and physical properties.

**Fig. 5** shows the storage stability in hexane in terms of the initial reaction rates for up to 4 days. The results for Novozyme 435® are superimposed. Lower activities for the fresh preparations are explained by the fact that separately prepared batches with batch-to-batch fluctuations in catalytic activity were used for the remaining data points. The data clearly shows that the solvent appears not to affect the subtle enzymatic catalysis for this stereoselective reaction substantially over the time investigated here. Unlike our previous work, where the catalytic mechanism does not require specific active site geometries; the active site's binding affinity is likely to remain unaffected during catalysis (Cao, 2005; Cruz et al., 2009).

**Fig. 6** shows the initial and residual activity of our CALB preparations after one year of storage at 4°C (glass vial, Teflon-lined screw cap). The activity of the 12 %SC and 4 %SC preparations is about 70% of the initial values (Table 2). The one year-stored 2 %SC preparation maintained over 90% of the same albeit low activity level of the fresh preparation (Table 2). As reported by (Cruz et al., 2009) this can be interpreted as evidence for stabilization due to enzyme-support interactions. The long term stability is encouraging in regard to practical application of the preparations.

# Conclusions

We extended the applicability of CALB biocatalysts immobilized on fumed silica to an enantioselective reaction in hexane. The catalytic activity of the biocatalysts is a function of the nominal surface coverage %SC by the immobilized enzyme molecules. A maximum in activity was found at a nominal surface coverage of approximately 230%SC, which confirms previous results for a conventional esterification. We hypothesize three different and to some extent overlapping enzyme surface loading regimes: 1. low nominal surface coverage where the surface-protein interactions dominate and deactivate many enzyme molecules; 2. intermediate nominal surface coverage where protein-protein interactions protect the catalytic activity of the enzyme molecules while access to the individual active sites is relatively unfettered; and 3. multi layer nominal surface coverage where enzymes are mass transfer limitations prevail. The preparation with nominal surface coverages of %SC 100 and %SC 230 showed better thermal stability at low specific catalytic activity which is perhaps due to the remaining active enzyme molecules' stabilization by strong surface-enzyme interactions. The enzyme/fumed silica preparation showed very good stability during prolonged exposure to hexane. Refrigerated storage of the preparation for one year caused only a 25% of reduction in the catalytic activity.

## Acknowledgements

Partial support for M. K. by the William H. Honstead Professorship in the Department of Chemical Engineering at Kansas State University is gratefully acknowledged.

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# Tables

**Table 1:**
Summary of the amount of fumed silica and aqueous buffer for the various enzyme preparations. In all cases, the amount of crude CALB was 5.83 mg.

| Target preparations (% nominal surface coverage) | Aqueous buffer [mL] | Fumed silica[a] [mg] | Enzyme concentration [mg/mL] | Amount of preparation containing 35 U[b] (mg of lyophilized preparation) |
|---|---|---|---|---|
| 2 | 58.3 | 651.3 | 0.1 | 131.4 |
| 4 | 58.3 | 325.7 | 0.1 | 66.3 |
| 12 | 19.4 | 108.6 | 0.3 | 22.9 |
| 17 | 8.3 | 76.6 | 0.7 | 16.5 |
| 50 | 2.1 | 26.1 | 2.7 | 6.4 |
| 100 | 1.7 | 13.0 | 3.5 | 3.8 |
| 150 | 1.5 | 8.7 | 3.9 | 2.9 |
| 230 | 1.5 | 5.7 | 4.0 | 2.3 |
| 300 | 1.2 | 4.3 | 4.7 | 2.0 |
| 400 | 1.2 | 3.3 | 5.0 | 1.8 |
| 538 | 1.1 | 2.4 | 5.1 | 1.7 |
| 1250 | 0.9 | 1.0 | 6.3 | 1.4 |
| 2087 | 0.9 | 0.6 | 6.3 | 1.3 |
| Novozyme 435® | - | - | - | 5.0 |

[a] the required amount of FS was calculated as follows: area FS = 3.32 $m^2$ / % SC (1)
amount FS = area FS x (g / 255$m^2$) (2)
where 3.32 $m^2$ is the area of 1.17mg enzyme regarding an area of 3.12 x $10^{-17}$ $m^2$/molecule

[b] the amount of preparation was calculated as follows:

$$\chi_{g\ prep} = \frac{Units\ CALB\ in\ preparation}{Units/g\ preparation} = \frac{35U}{Units/g\ preparation} \quad (3)$$

$$Units/g\ preparation = \frac{Units\ weighed\ CALB}{g\ CALB + g\ FS} \quad (4)$$

**Table 2:**
Long-term stability (reaction conditions: 30°C, 6 mL reaction volume, 35U of enzyme per preparation) of various CALB/fumed silica preparations (storage at 4°C, closed glass vials).

| Preparation [%SC] | $r_0$ fresh preparation [μmol*min$^{-1}$] | $r_0$ one year-aged [μmol*min$^{-1}$] | Activity retained after one year storage |
|---|---|---|---|
| 2 | 0.41 | 0.37 | 90 % |
| 4 | 0.50 | 0.35 | 70 % |
| 12 | 0.88 | 0.64 | 73 % |

# List of Figures

**Fig. 1.** Time evolution of the fractional conversion of (R)-1-phenylethanol during the transesterification with vinyl acetate for CALB/FS preparations with different nominal surface coverages %SC (30°C, 48 mL reaction volume, 35U of enzyme per preparation): 4 %SC (●), 12 %SC (□), 17 %SC (■), 100 %SC (△), 230 %SC (◊), 400 %SC (◆).Novozyme 435® during the initial 1.33 hours of reaction (X). Reverse reaction carried out with 230 %SC preparations (-); Solid lines added to guide the eye; *y*-error bars show the cumulative standard errors and are obtained from the kinetic parameter calculations.

**Fig. 2.** Catalytic activity of CALB/FS preparations as a function of the nominal surface coverage %SC. (◊) Enantioselective transesterification of (RS)-1-phenylethanol in n-hexane. (■) Esterification of geraniol with acetic acid in n-hexane. (---) Reaction rate for Novozyme 435® in the enantioselective transesterification of (RS)-1-phenylethanol; (···) represents Novozyme 435® in geraniol system. *y*-error-bars and short dashed lines represent the cumulative standard error from the calculation of the initial reaction rates. (30°C, 6 mL reaction volume, 35U of enzyme per preparation).

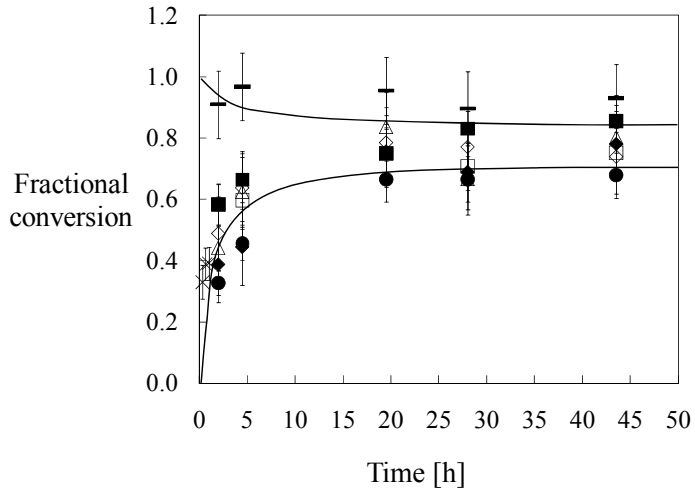
**Fig. 3.** Schematic of the three regimes controlling the catalytic activity per enzyme molecule of FS/CALB preparations in hexane: 1. at low surfaces coverages, interactions with the surface are maximized leading to detrimental conformational changes. 2. at intermediate surface coverages, a transitional regime where enzyme structure is more generally maintained. 3. at high surface coverages multi-layer coverage leads to mass-transfer resistance and low apparent catalytic activity per enzyme molecule.

**Fig. 4.** Temperature stability of CALB/FS preparations with various surface coverages (%SC): 2 %SC (○), 12 %SC (□), 100 %SC (Δ), 230 %SC (◊). Temperature stability of Novozyme 435® preparations (X). (▲) *R. miehei* lipase immobilized in agar organogels based on lecithin, isooctane as solvent, shown for comparison (Zoumpanioti et al. 2008) Solid lines added to guide the eye; *y*-error bars show the cumulative standard errors from the kinetic parameter calculation. (Various temperatures, 6 mL reaction volume, 35U of enzyme per preparation).

**Fig. 5.** Solvent stability of CALB/FS preparations with various surface coverages %SC. After 4 days exposure to the solvent, the catalytic activity remains almost unaffected: 400 %SC (◆), 230 %SC (◊), 100 %SC (Δ), 2 %SC (○). Storage stability of Novozyme 435® in n-hexane (X). *y*-error bars represent the cumulative standard error and are obtained from the initial reaction rate calculation. (30°C, 6 mL reaction volume, 35U of enzyme per preparation).

**Fig. 6.** Long-term stability of CALB/FS preparations with various surface coverages %SC: (◊) fresh preparations, (□) one year-aged preparations (storage at 4°C, closed glass vials). Cumulative standard errors are obtained from the kinetic parameter calculation and are shown as *y*-error bars. (30°C, 6 mL reaction volume, 35U of enzyme per preparation).

**Fig. 1**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0.6
0.4
0.2
0.0
Fractional conversion
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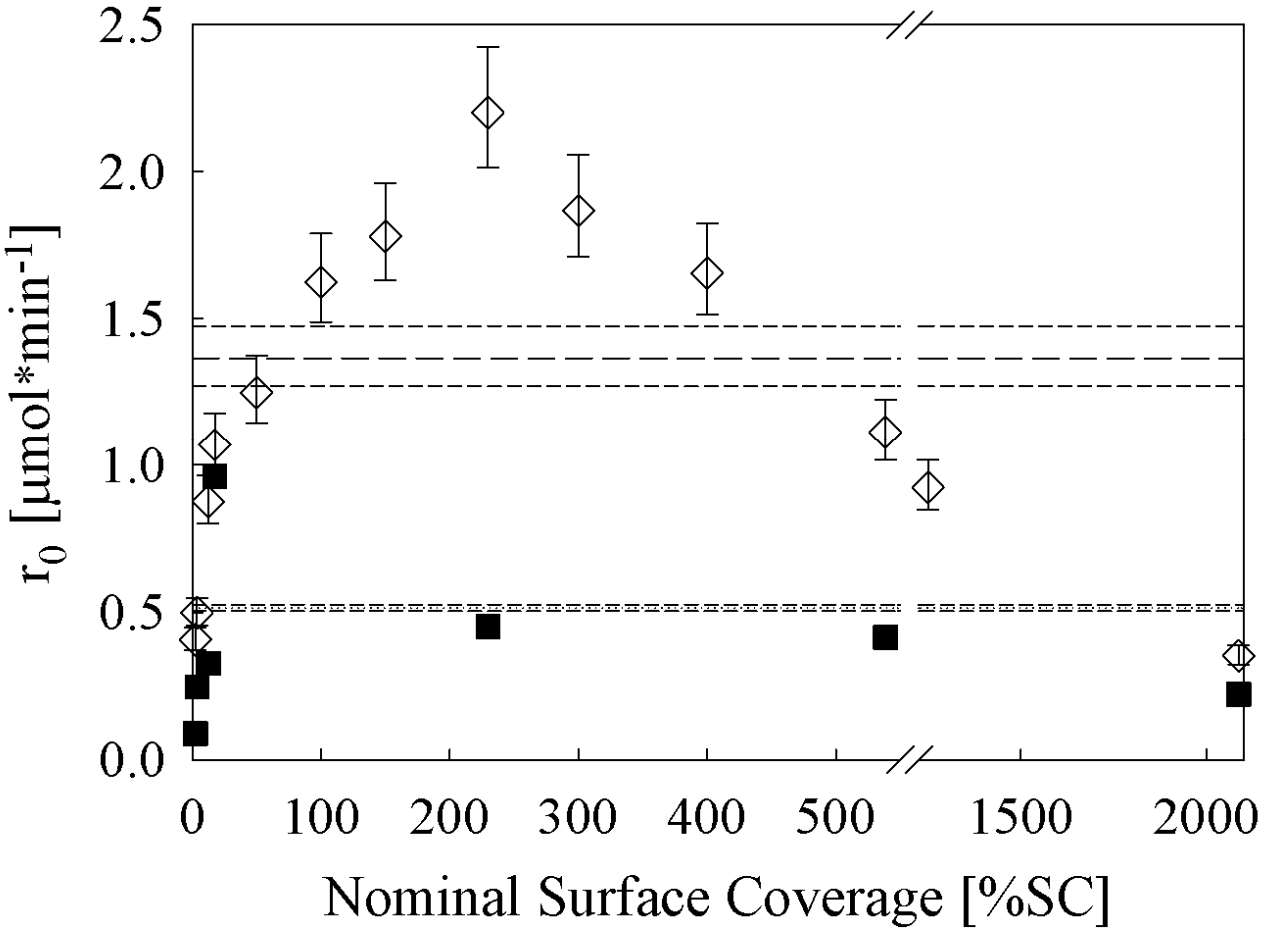
5
10
15
20
25
30
35
40
45
50
Time [h]

**Fig. 2**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1.5
1.0
0.5
0.0
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100
200
300
400
500
1500
2000
Nominal Surface Coverage [%SC]

Fig. 3

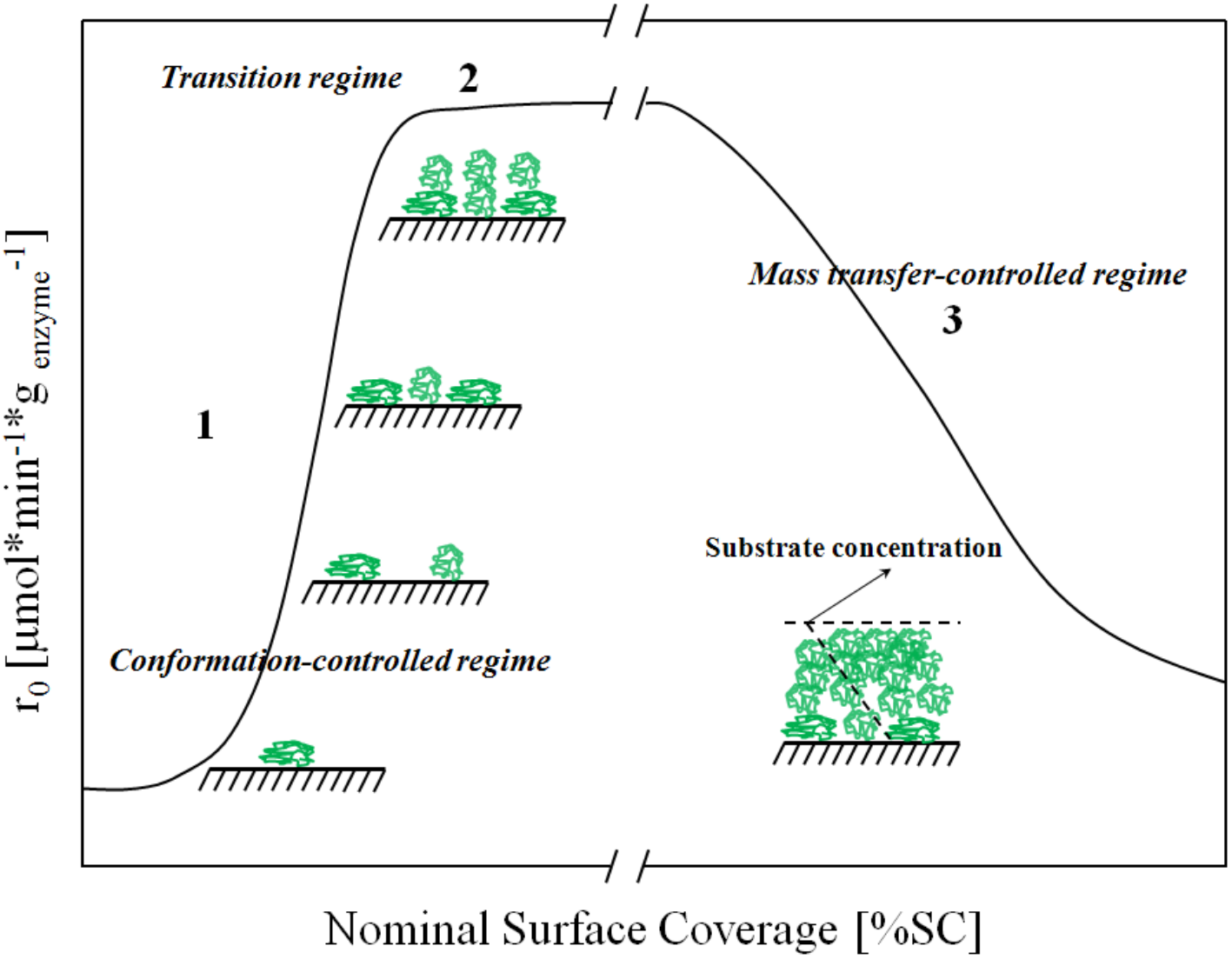

Transition regime
2
Mass transfer-controlled regime
3
1
Substrate concentration
Conformation-controlled regime
r0 [µmol*min-1*g enzyme-1]
Nominal Surface Coverage [%SC]

**Fig. 4**

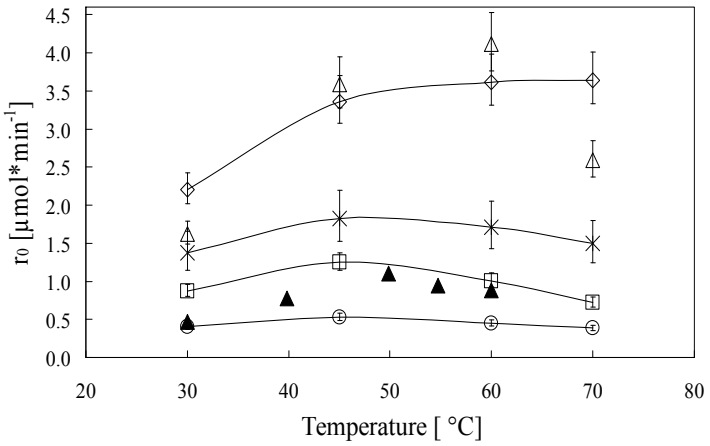

4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
r0 [µmol*min-1]
20
30
40
50
60
70
80
Temperature [ °C]

**Fig. 5**

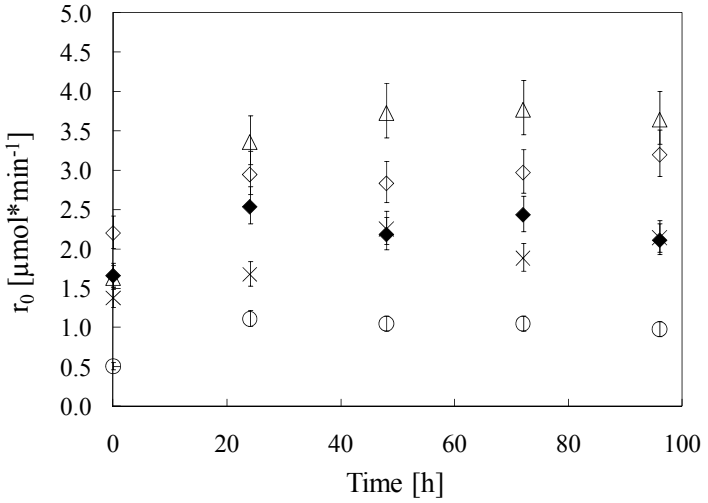
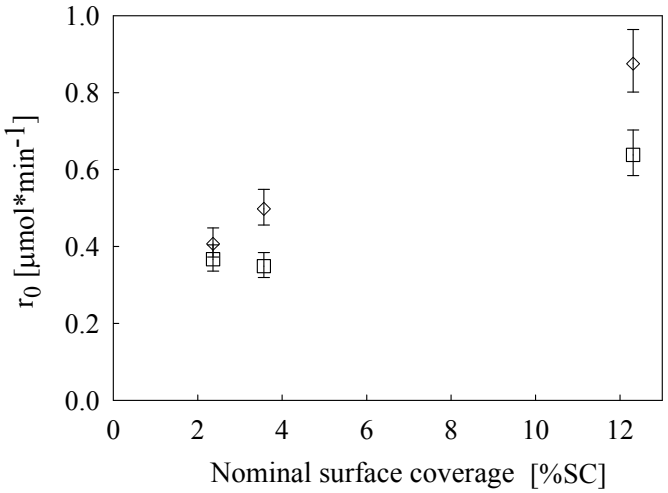


**Fig. 6**


1.0
0.8
0.6
0.4
0.2
0.0
$r_0$ [µmol*min$^{-1}$]
0
2
4
6
8
10
12
Nominal surface coverage [%SC]