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Effects of supercritical CO_2 exposure and depressurization on immobilized lipase activity

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Abstract

Lipozyme IM20 from Novo Nordisk (Denmark) was examined after various treatments. Conditions were chosen to reflect those that would be considered in the design of an industrial process. A two-level factorial design was employed to assess the effects of pressurization/depressurization cycles, rate of depressurization and exposure length. A significant three-factor interaction was observed. Lowest residual activity was observed for runs in which the depressurization rate was 86–89 bar min^{-1}. Incubation for 12 h also yielded low residual activity but only when exposing the immobilized enzyme to one cycle. The highest residual activity was obtained for immobilized enzymes repeatedly exposed for periods of 12 h (5 times) with a depressurization rate of 4.3 to 4.45 bar min^{-1}. This effect may be due to the extraction of an inhibiting compound. Tuning process parameters can lead to a seven-fold change in residual activity.

Introduction

The stability of a biocatalyst can be key in the design, operation and success of an industrial biotransformation process. Interest has developed in the use of supercritical carbon dioxide (SC CO_2) as a reaction medium for enzymes since the discovery that enzymes remain active under such conditions. Several advantages include low solvent toxicity, enhanced mass transfer, and the ability to control solubility of solutes with pressure manipulation.

Since the first reports by Hammond *et al.* (1985), Randolph *et al.* (1985) and Nakamura *et al.* (1986) on enzymatic reactions in SC CO_2, little work has been done to understand the interaction of different operating conditions. Although considerable research has focused on assessing the stability of enzymes based on residual activity determinations, the conditions under which the enzymes have been treated vary enormously. From an engineering perspective, it is difficult to get a sense of how certain processing parameters affect enzyme stability.

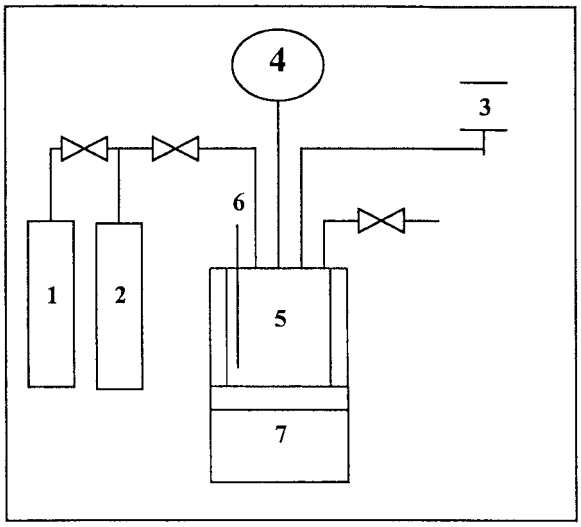

Fig. 1. Schematic of experimental reactor set-up. (1) Extraction grade CO_2; (2) practical grade CO_2; (3) pressure relief valve; (4) pressure gauge; (5) 300 ml quartz windowed reactor; (6) thermocouple; (7) magnetic stirrer.

Table 1. List of reagents for activity determination based on free fatty acid analysis.

Reagent	Description
1	1% (w/v) olive oil in ethanol
2	0.025 M Tris buffer containing 2 g deoxycholic acid l^{-1} (pH 8.8)
3	Substrate emulsion 7.5 ml of reagent **1** added to 500 ml of reagent **2** while stirring vigorously with a magnetic stirrer
4	0.27 M cupric nitrate in 0.45 M triethanolamine buffer (pH 10.4)
5	9 mM diethyldithiocarbamate in butanol

Table 2. Design matrix for 2^3 factorial experiment.

Run	Number of pressurization/ depressurization cycles	Rate of depressurization	Length of exposure
1	−	−	−
2	−	+	−
3	+	−	−
4	+	+	−
5	−	−	+
6	−	+	+
7	+	−	+
8	+	+	+
9	0	0	0
10	0	0	0
11	0	0	0

The purpose of this study was to quantify the effects of three parameters, relevant to industrial applications, by performing a full two-level factorial experiment. The three parameters that were investigated were the rate of depressurization, the length of incubation in SC CO_2 and the number of pressurization/depressurization cycles (herein referred to as the number of exposures).

Materials and methods

Enzyme source

Lipozyme IM20, a lipase from *Rhizomucor miehei,* immobilized on a macroporous anion exchange resin, was a kind gift from Novo Nordisk (Denmark) and was used for all the experiments.

Determination of residual activity

The residual lipase activity was determined using a photometric method by Verduin *et al.* (1973). The method was slightly modified to accommodate the assay of immobilized enzymes and was based on the hydrolysis of olive oil.

The reagents used in the analysis are listed in Table 1. The chemicals used included extra virgin olive oil, deoxycholic acid (sodium salt), sodium diethyldithiocarbamate (certified A.C.S.), cupric nitrate (certified A.C.S.), HPLC grade 1-butanol, and triethanolamine, ethanol, Tris, and HPLC grade chloroform.

In a reaction vessel, 50 ml reagent **3** was combined with 0.5 g immobilized enzyme at 25 °C. Three ml samples were added to 2 ml reagent **4**, which terminated the reaction and converted the liberated fatty acids to their copper salts. The copper salts were extracted from the aqueous phase with 8 ml chloroform. After vigorous shaking the mixture was centrifuged at 1500 *g* for 5 min to separate the emulsion. Three ml organic phase (lower phase) was reacted with 0.3 ml reagent **5** to form an orange compound. The absorbance was read at 520 nm.

Exposure to supercritical carbon dioxide

Immobilized enzymes were contained in a nylon cloth bag (25 μm mesh) for easier manipulation prior to putting them in the reactor. After flushing the reactor with a practical grade CO_2 the reactor was pressurized using SCF/SFE grade CO_2. Figure 1 is a schematic of the experimental set-up.

Temperature was monitored with a thermocouple and controlled using a thermostat and electrical heating tape. Conditions were 35–37 °C at a pressure between 86 and 89 bar. Supercritical conditions were assessed visually through quartz viewing windows on the reactor. A homogeneous environment within the reactor was ensured by stirring with a magnetic stir bar. Temperature was allowed to restabilize after depressurization before repressurizing the reactor.

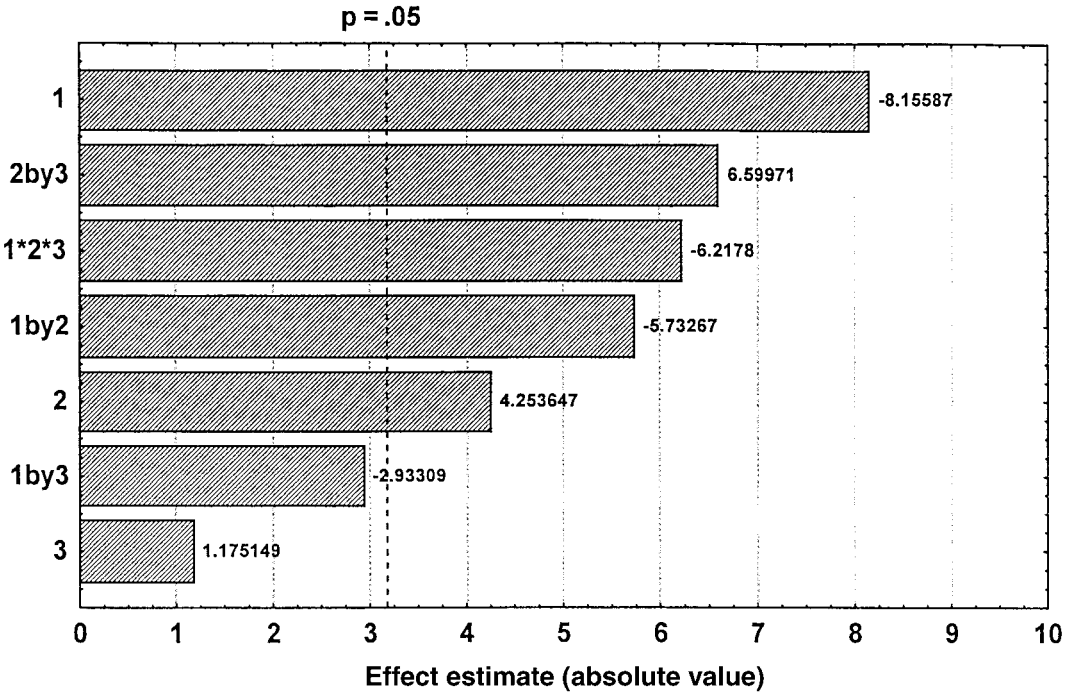

Fig. 2. Pareto chart of standardized effects for 2^3 factorial experiment (MS residual = 0.000004). (1) Depressurization rate; (2) number of exposures; (3) length of exposure.

Factorial design

Three centerpoints designated as 0,0,0, were added to the factorial design to determine the error associated with the reproducibility of the data. A total of 11 runs were performed. Table 2 summarizes the different combinations of parameters to obtain the full factorial design. The three levels of pressurization/depressurization cycles correspond to 1, 3 and 5 cycles. The three levels of rates of depressurization correspond to 86–89 bar min^{-1} (depressurized the reactor in 1 min), 8.6–8.9 bar min^{-1} (depressurized the reactor in 10 min) and 4.3–4.45 bar min^{-1} (depressurized the reactor in 20 min). The three exposures were for 4, 8 and 12 h. The runs were run in random order to avoid any biased results. Results were analyzed using STATISTICA (Release 5.1) by StatSoft, Inc. (Tulsa, Oklahoma, USA).

Results and discussion

The three parameters investigated were the rate of depressurization, the number of exposures and the length of exposure in SC CO_2. To examine these parameters, the residual activities of the enzymes after exposure to SC CO_2 were measured. The residual activity ranged from 5.35 to 38 μmol fatty acid/(min g immobilized enzyme). The standard deviation based on the center point measurements was 2.29 μmol fatty acid/(min g immobilized enzyme).

The Pareto chart of effects (Figure 2) shows the standardized effects of the parameters and their interactions. The individual effects of rate of depressurization, the number of exposures and the length of exposure in SC CO_2 cannot be interpreted separately because of the significant $1\times2\times3$ interaction (at a 95% confidence level).

When comparing Figures 3A and 3B, it can be seen that a high depressurization rate (Figure 3B) is detrimental to the biocatalyst. This remains true even when manipulating the other two processing variables in this study. The faster the depressurization of the reactor, the greater the negative effect was on the residual activity of the enzyme, that is reduced residual activity. The differences in Figures 3A and 3B also show that with a slow rate of depressurization, the effects of the other parameters become more pronounced. The rate of depressurization has an effect on the activity of the biocatalyst. It can be seen that simply tuning the depressurization rate and keeping the other two

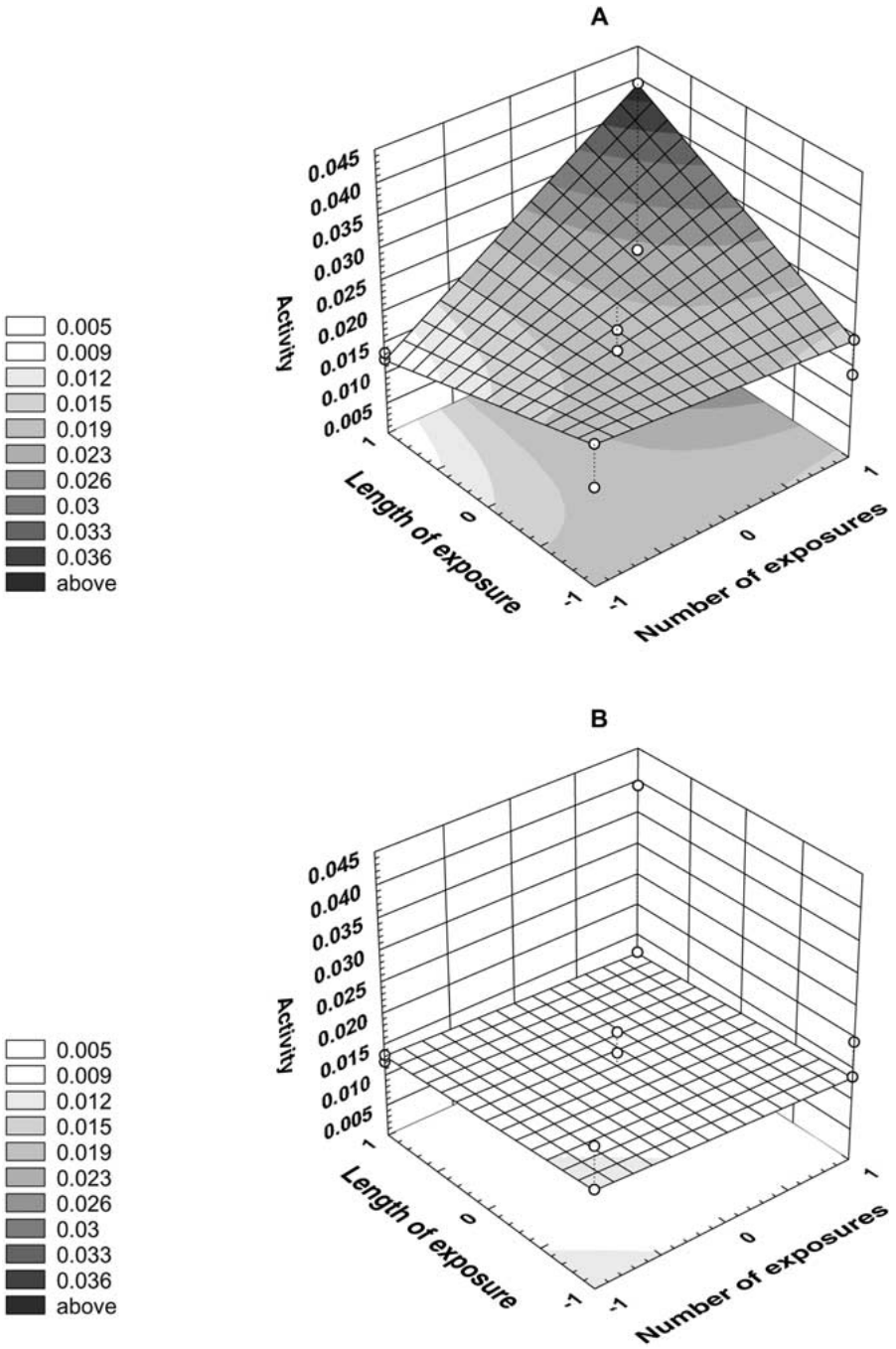

Fig. 3. Surface plots of activity (mmol liberated fatty acid min^{-1} g^{-1} immobilized enzyme) for 2^3 factorial experiment (MS residual = 0.000004). (A) Depressurization rate = 4.3–4.45 bar min^{-1}. (B) Depressurization rate = 86–89 bar min^{-1}.

parameters constant can increase the residual activity six-fold. There are a number of forces acting on the enzyme and the immobilization matrix during the depressurization step, which may cause pore collapse and/or modifications to the conformational structure of the enzyme. The conformational changes could be attributed to the rate of escape of the dissolved CO_2 from the bound water surrounding the enzyme as speculated by Kasche *et al.* (1988) but there are inherent diffi-

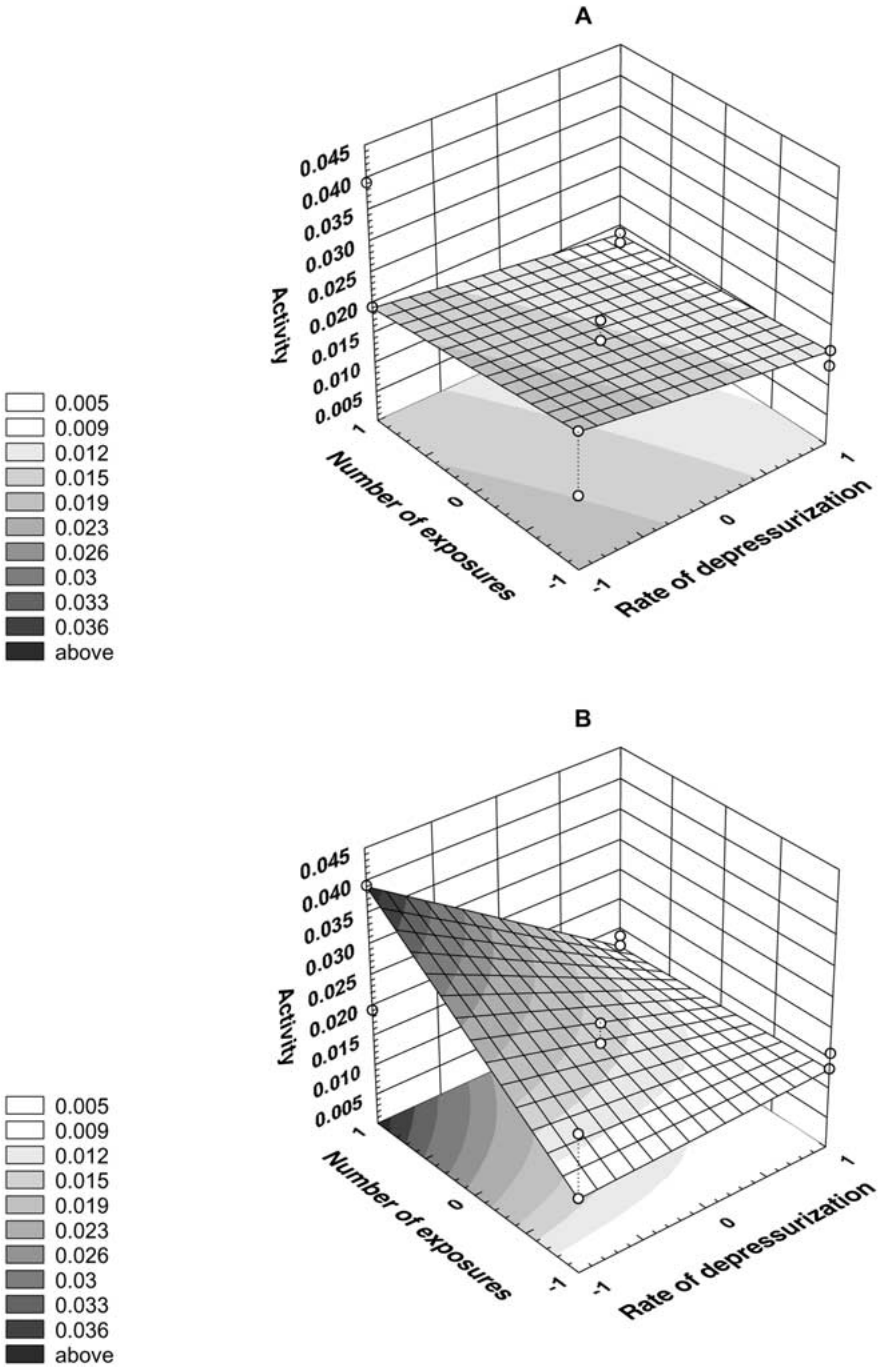

Fig. 4. Surface plots of activity (mmol liberated fatty acid min^{-1} g^{-1} immobilized enzyme) for 2^3 factorial experiment (MS residual = 0.000004). (A) Length of exposure = 4 h. (B) Length of exposure = 12 h.

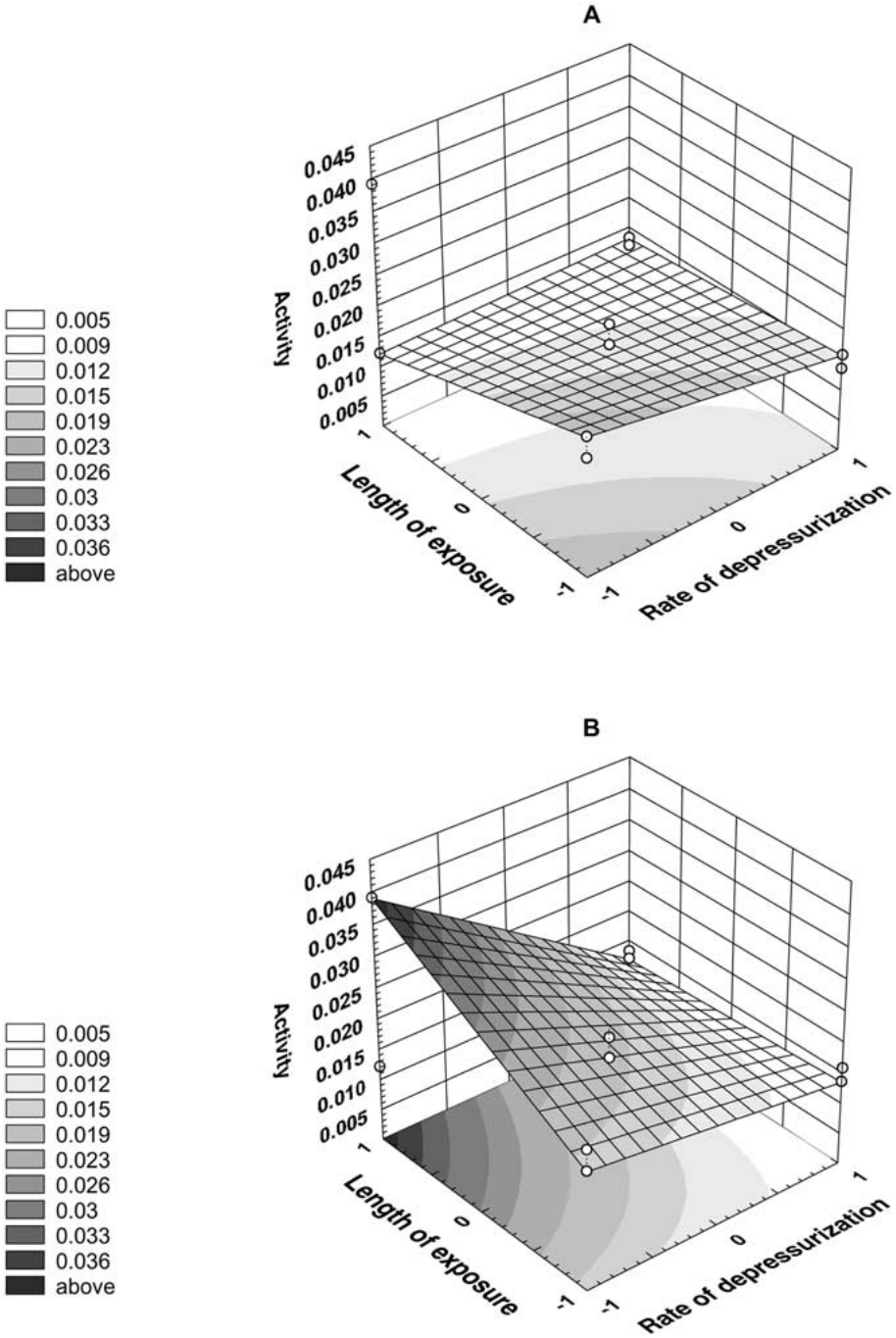

Fig. 5. Surface plots of activity (mmol liberated fatty acid min^{-1} g^{-1} immobilized enzyme) for 2^3 factorial experiment (MS residual = 0.000004). (A) Number of exposures = 1. (B) Number of exposures = 5.

culties in verifying this hypothesis with immobilized enzymes.

Zagrobelny & Bright (1992) would argue this point as they demonstrated through steady-state fluorescence spectroscopy that the conformational change in trypsin occurring during exposure to carbon dioxide was a result of the compression step and not the decompression step. Gießauf *et al.* (1999) reported rates of depressurization (10 to 21 bar min^{-1}), but did not account for the rate of depressurization as a factor influencing the residual activity.

Some earlier work, though, has reported that for depressurization rates between 10 and 20 bar min^{-1}, rates slightly larger than our intermediate value, repeat depressurizations in dry SC CO_2 have a beneficial effect especially for bovine trypsin (Kasche *et al.* 1988). Zheng & Tsao (1996) reported that to prevent the loss of residual cellulase activity, the rate of depressurization needed to be smaller than that reported by Kasche *et al.* (1988). Kamat *et al.* (1992) noted that enzymes retain activity after slow depressurization from supercritical conditions but did not specify a rate. These previous results are in agreement with the ones presented in this paper.

Figures 4A and 4B show the change in activity at the low and high levels of duration of exposure. It can be seen that as the incubation length is increased the number of exposures becomes a significant parameter in increasing the residual activity. The number of exposures can cause close to a five-fold increase in residual activity when exposing the immobilized enzymes to 12-h incubation periods and slow rates of depressurization (4.3–4.45 bar min^{-1}). The effect of these parameters may be linked to the extraction of material from the matrix that may inhibit enzyme activity or access of the substrate to the enzyme. Al-Duri *et al.* (2001) have recently shown that by exposing Accurel EP100 to SC CO_2, the matrix changed slightly to yield a material with a slightly larger pore volume and surface area. The effect observed here, may therefore also be related to a physical change in the support matrix by providing better accessibility to the active site of the biocatalyst.

The extraction properties of SC CO_2 are well known and have been reviewed recently by Marr & Gamse (2000). Gießauf & Gamse (2000) have also demonstrated the extraction capabilities of SC CO_2 through the extraction of fatty acids from a crude preparation of porcine pancreatic lipase. We have made similar observations in our own work in biotransformations using whole cells in SC CO_2 (Ng & Legge, unpublished).

Figures 5A and 5B show the results at a low and high level of number of exposures, respectively. These plots suggest that a long period of exposure seems to be detrimental to the residual activity when the enzyme is only incubated once. When the immobilized enzymes are repeatedly incubated for 12 h periods with slow rates of depressurization, an optimum residual activity (solely based on the range defined in this study) is obtained. This phenomenon could be explained through a dual mechanism. Simple exposure to SC CO_2 may have a detrimental effect on the protein. Weder (1984) reported protein (lysozyme and ribonuclease) unfolding and partial oligomerization when subjected to SC CO_2. Repeated exposures though, could enhance changes in the support matrix due to the dissolution and extraction of material. It is believed that observed activity is a balance between detrimental changes to the enzyme and positive changes to the catalytic microenvironment.

In conclusion, Lipozyme IM20 showed an optimum increase in activity after repeat exposures to SC CO_2 and low depressurization rates. It was observed that all the three parameters investigated affect the residual activity of the enzyme. It is hypothesized that the increase may be due to an extraction of inhibiting components in the immobilization matrix. Quantitatively, the rate of depressurization has a negative effect on the residual activity and that it is beneficial to depressurize at a rate less than 8.9 bar min^{-1}.

Acknowledgements

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