Changes in Enzyme Efficiency During Lipase-Catalyzed Hydrolysis of Canola Oil in a Supercritical Bioreactor

Rezaei, Karamatollah*+

Department of Food Science & Engineering, Faculty of Agricultural Biosystem Engineering, University of Tehran, P.O. Box 31587-78659 Karaj, I.R. IRAN

Temelli, Feral

Department of Agricultural, Food and Nutritional Science, University of Alberta Edmonton, Alberta, T6G 2P5 CANADA

ABSTRACT: *Enzyme efficiency was investigated in the lipase-catalyzed hydrolysis of canola oil in supercritical carbon dioxide ($SCCO_2$). Immobilized lipase from Mucor miehie (Lipozyme IM) was used as the catalyst and the results showed that enzyme efficiency dropped at high pressures indicating a possible change in enzyme microstructure. Therefore, scanning electron microscopy (SEM) was used as a supplementary tool to investigate microstructural stability of the enzyme under supercritical conditions. SEM images of the treated enzymes did not demonstrate any apparent structural changes with a change in pressure (24.0 and 38.0 MPa), enzyme load of the reactor (1.0 and 5.0 g), CO_2 flow rate (0.5 and 3.9 L/min) and the oil content (extracted from 3.0 and 15.0 g canola flakes) of $SCCO_2$. However, a change at the molecular level is a possibility, which requires further investigation.*

KEY WORDS: *Canola oil, Biocatalysis, Bioreactor, Enzyme efficiency, Hydrolysis, Microstructure, Scanning electron microscopy, Supercritical*.

INTRODUCTION

The applicability of supercritical fluids to the fast growing enzyme technology has provided an alternative to the use of organic solvents to conduct enzymatic reactions. Supercritical carbon dioxide ($SCCO_2$) is the most commonly used supercritical solvent for food applications in research and industry [1-8]. Enzymes may loose part of their activity when exposed to high temperature, excessive moisture content and/or high pressures [9]. Enzyme inactivation is not necessarily due to some physical structural changes. *Kamat et al.* [10] using Laser Desorption Mass Spectrometry (LD/MS) reported that CO_2 under supercritical conditions formed a

* *To whom correspondence should be addressed.*
+ *E-mail: krezaee@ut.ac.ir*
1021-9986/06/4/25 11/$/3.10

covalent bond with lipase and inhibited its activity. Although various studies [1,11,12] have investigated the effect of pressure and other parameters such as temperature, water content and type of the supercritical fluid on the enzyme stability, our understanding of structural changes that may occur when an enzyme is exposed to high pressure in supercritical media is quite limited. A loss in enzyme activity and/or stability after exposure to certain conditions such as high pressure [1,8,11,12] or excessive water content [9] has been reported, in which cases the extent of inactivation had a direct negative effect on the reaction process in terms of reaction rate and the amount of products, etc. On the other hand, there are reports claiming an increase in enzyme activity with increasing pressure [13]. It is well known that enzymes exposed to ultra high pressures (>400 MPa) undergo irreversible structural changes, which will result in complete inactivation of the enzyme [14].

Scanning Electron Microscopy (SEM) is a typical approach for morphological studies [15-19]. Since enzymes vary in size, their structure may appear differently when observed under an electron microscope. Smaller enzymes can stay very close to each other during immobilization and therefore a higher magnification level will be required to have a better image of the enzyme structure. On the other hand, larger enzymes can be easily visible when using an electron microscope at a lower magnification level. For example, while the enzyme *Aspergillus* catalase was hardly visible under an electron microscope at 7,000X magnification, bovine liver catalase could be easily detected at 560X magnification [15]. This made the authors to be curios about the possibility of viewing certain changes that might happen in the enzyme structure when exposed to high pressures in a biocatalytic reaction. The incentives of such studies were many reports in the literature [1,8-12] as to the loss of enzyme performance during a relatively long enzyme-catalyzed reaction. The authors own experience with immobilized enzyme from *Mucor miehei* clearly showed that the enzyme had lost its capability to catalyze the hydrolysis of canola oil continuously supplied to the enzyme sites during a 24-h reaction under $SCCO_2$ environment (Fig. 1). Despite many reports in the literature for the application of SEM to investigate changes in the enzyme structure, there is no report indicating the use of this approach to analyze the microstructure of immobilized enzymes exposed to the range of conditions typical to supercritical studies (≤40 MPa). Therefore, the objectives of this work were to study the effects of pressure, CO_2 flow rate, enzyme load and oil content on the enzyme efficiency and also to correlate the results with possible microstructural changes in the enzyme (immobilized lipase from *Mucor miehei*).

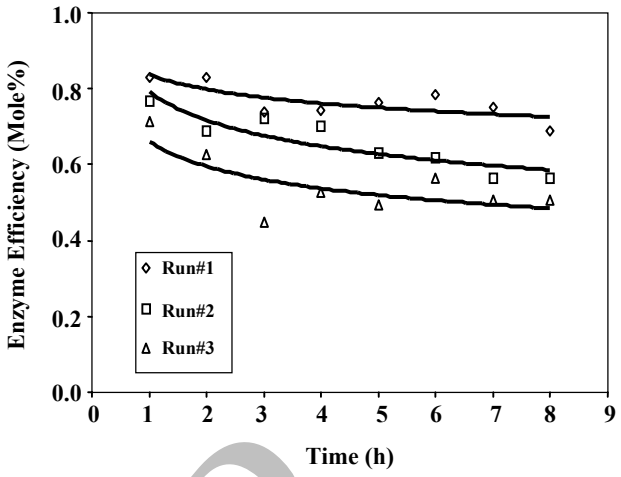

Fig. 1: Changes in enzyme efficiency during three consecutive 8-h runs using a single batch of enzyme in a continuous-flow lipase-catalysed hydrolysis in supercritical carbon dioxide at 38.0 Mpa and 55° C [enzyme (1.0 g): Lipozyme IM, immobilized lipase from Mucor miehie; CO_2 flow rate: 3.7 L/min].

MATERIALS AND METHODS

Materials

Cooked canola, *Brassica napus* and *B. campestris,* flakes and *Mucor miehei* lipase originally immobilized on macroporous anionic resin (size range: 0.2-0.6 mm), Lipozyme IM, were kindly provided by CanAmera Foods (Fort Saskatchewan, AB) and Novo Nordisk (Franklinton, NC), respectively. The enzyme was stored at 5°C until used in the experiments. CO_2, used as extraction solvent and also as reaction medium (bone dry, 99.8%), was purchased from Praxair (Mississauga, ON).

Experimental setup and design

A laboratory scale supercritical extraction system (Newport Scientific, Inc., Jessup, MD) was modified to conduct on-line extraction and/or hydrolysis of canola oil in $SCCO_2$ (Fig. 2). A new stainless steel extraction cell (13 cm × 29 mm I.D.) was fabricated and positioned after the compressor. Water was introduced using a piston

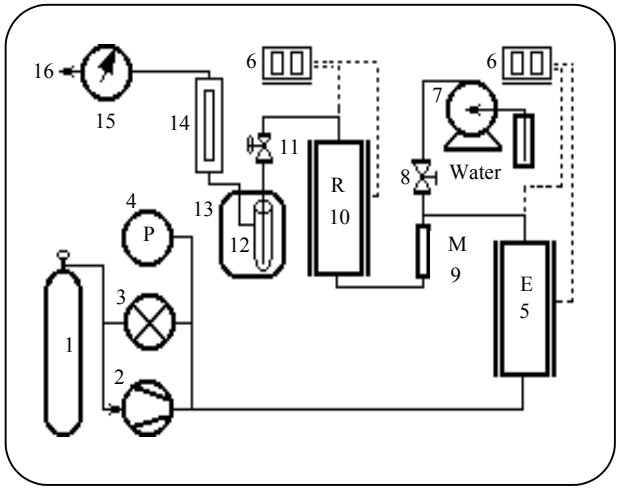

Fig. 2: Schematic of the on-line supercritical extraction and enzymatic-reaction system. 1- CO_2 tank, 2- Compressor, 3- Back-pressure regulator, 4- Pressure gauge, 5- Extraction chamber, 6- Temperature control, 7- Piston pump, 8- On/off valve, 9- Mixer, 10- Reaction (enzyme) cell, 11- Depressurization valve, 12- Sample collection tube, 13- Cold bath, 14- Rotameter, 15- Gas meter, 16- Vent.

pump (Gilson 305, Middleton, WI) equipped with a manometric module (Gilson 805, Middleton, WI) and mixed with oil-laden CO_2 exiting the extractor. A mixer was assigned close to the bottom of the reaction cell, where oil-laden CO_2 and water were mixed prior to entry into the reactor. A small stainless steel reaction cell (15 cm × 13 mm I.D.) was made and placed inside the original extraction cell. Immobilized enzyme beads were mixed with glass beads (7.9 g, 3.76 mm average O.D.) to prevent formation of a compact enzyme bed. The mixture of enzyme and the glass beads was positioned in the top portion of the reaction cell and the lower portion of the cell as well as the space above the enzyme layer were filled with glass wool. The extraction and reaction temperatures were measured using two separate thermocouples held within the top portions of the respective chambers, and maintained within ±2°C of the desired temperatures with separate controllers. Pressure was maintained by a back-pressure regulator. Reaction product samples were collected into two successive side-armed test tubes held in a cold bath at -20°C after depressurization. CO_2 passed through a flow-meter and a gas-meter before it was vented.

Experiments were conducted at two different pressures (24.0 and 38.0 MPa) and 35°C. CO_2 flow rate (0.5 and 3.9 L/min, measured at ambient conditions), level of enzyme load (1.0 and 5.0 g) and amount of canola flakes (3.0 and 15.0 g) used as the source of canola oil were the parameters studied. Reactions were continued for 6 h and then enzyme batches were taken out of the reactor after a slow depressurization (~30 min) and stored at 5°C until analyzed by SEM. The reaction products were also analyzed for their free fatty acids, monoglycerides (MG), diglycerides (DG), triglycerides (TG) and glycerol following a method described elsewhere [8]. The term *enzyme efficiency* was applied to characterize enzyme capability in hydrolyzing TG into glycerol, which was determined based on the hydrolyzed fraction of the original TG introduced to the enzyme sites. For partial conversions of TG into DG and MG, 1/3 and 2/3, respectively, of a full hydrolysis were considered.

Sample preparation for scanning electron microscopy

Typical representatives of different batches of enzyme used at various studied reaction conditions were selected for SEM analysis. A JEOL 6301 FXV scanning electron microscope (Peabody, MA) was used for the analysis of the immobilized lipase before and after catalyzing the reactions in $SCCO_2$ media. Two different coating methods were applied for sample preparation. In the first method, each enzyme grain was fixed on a specimen-mounting stub using a double-sided conductive carbon tape and then gold-sputtered in argon atmosphere using a Nanotech Semprep 2 system (Neuilly, France). In the second method, enzyme grains were attached to the SEM stubs using conductive silver paint, which was allowed 5 days to dry before use. Then, the grains were sputter-coated with chromium in xenon atmosphere using an Edward high-vacuum XE 200 Xenosput (Crawley Sussex, England) and analyzed. The second method was used to achieve a better enzyme attachment to the specimen mounting stubs so that the enzyme grains would not move during SEM analysis. In both methods, some moving of the samples was observed occasionally and results from samples with severe moving problems were not included for the discussion.

RESULTS AND DISCUSSION

SEM images of the samples at various magnification levels were obtained to examine possible structural

changes. However, enzyme from *Mucor miehei* in this study could not be observed on the micrographs at low magnification levels. Therefore, all of the images were reported at 30,000X magnification level. Neither the free enzyme (i.e. enzyme not immobilized on any support) nor the enzyme-free anion exchange resin was available for comparison. However, based on the SEM images at 30,000X magnification obtained in this study, the spherical particles ranging in size 35-55 nm on the outside surface of the solid support were interpreted to be a group of enzyme molecules.

Effect of high pressure

Changes in the enzyme efficiency at two different pressures (24.0 and 38.0 MPa) during a continuous-flow hydrolysis of canola oil in $SCCO_2$ are shown in Fig. 3. Significant changes in the enzyme efficiency were observed due to the change in pressure. Although, according to *Miller et al.* [20], pressure at the levels of this study does not have significant effect on the enzyme activity, certain changes in enzyme efficiency can be attributed to the changes in the medium properties such as phase behavior, diffusion properties and therefore substrate concentrations on the enzyme sites. However, when a continuous drop in the enzyme efficiency is observed at given operational conditions, then it has to be related to possible changes in the enzyme behavior due to a change in its (micro)structure. The micrographs of 1.0 g enzyme batches undergone a 6-h run at 24.0 and 38.0 MPa and 35°C in $SCCO_2$ were compared to those of the untreated enzyme in Fig. 4a,b,c,d,e,f. In these experiments, oil was extracted from 15.0 g canola flakes with CO_2 at a flow rate of 3.9 L/min and water was pumped into the oil-laden CO_2 at a rate of 0.004 mL/min. Results from the two different sputtering methods applied are presented. Micrographs on the left column of Fig. 4 are from gold-sputtered samples and the ones to the right are from chromium-sputtered samples. In this study, inactivation of the enzyme by water is a possibility. However, there are no visible differences attributable to the effect of pressure on the microstructure of the enzyme. Similar to this study, *Vasudevan* and *Weiland* [15] were not able to observe any apparent changes in the SEM micrographs of bovine liver catalase inactivated by 0.01-1.0 M hydrogen peroxide. There was no pressure involved in their study.

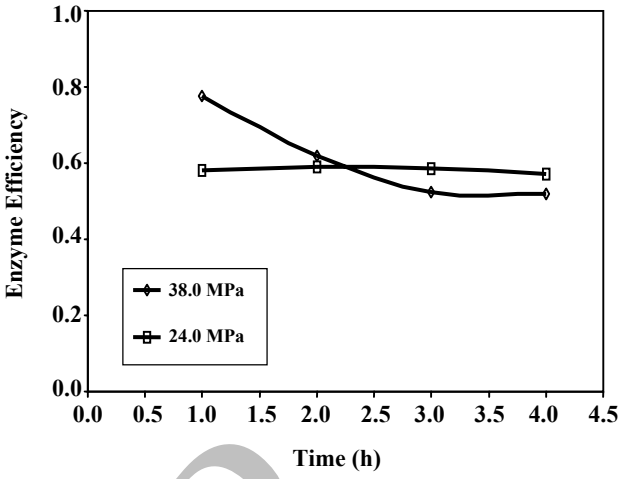

Fig. 3: Effect of pressure on the enzyme efficiency during a 4-h continuous-flow enzymatic hydrolysis of canola oil in supercritical carbon dioxide at 24.0 and 38.0 MPa and 35° C [enzyme (1.0 g): Lipozyme IM, immobilized lipase from Mucor miehie; CO_2 flow rate: 3.7 L/min].

Along to the above results, *Balaban et al.* [21] treated orange juice samples containing their natural pectin-esterase with $SCCO_2$ at 13.7 and 31.0 MPa and 40-60°C and reported a decrease in pectinesterase activity to a level at which the orange juice maintained its cloudy appearance longer than when it was untreated. Although temperature was a factor in reducing pectinesterase activity, it was not significant if high pressure were not applied [21]. The treatment with high pressure CO_2 involved reducing pH due to the formation of carbonic acid from the dissolution of carbon dioxide in water. The orange juice exhibited pH's as low as 2.96 at 31.0 MPa and 35°C. *Balaban et al.* [21] suggested that the low pH might be the main parameter in reducing pectinesterase activity. However, there are some enzymes that are active at pH's as low as 2.0. For example, lipase from *Candida cylindracea* is active over the pH range of 2.0-8.5 [15]. In addition, *Owusu-Yaw et al.* [22] showed that only when the pH of orange juice was dropped to ~2.0 by the addition of HCl or when the orange juice was treated with acidic cationic resins, could substantial reduction in pectinesterase activity take place.

Those results along with the results of this study indicate that the enzyme inactivation is not at a level to be observed by physical structural analysis like the one performed by SEM. However, there could be some chemical changes occurring at the inter- or intra- molecular

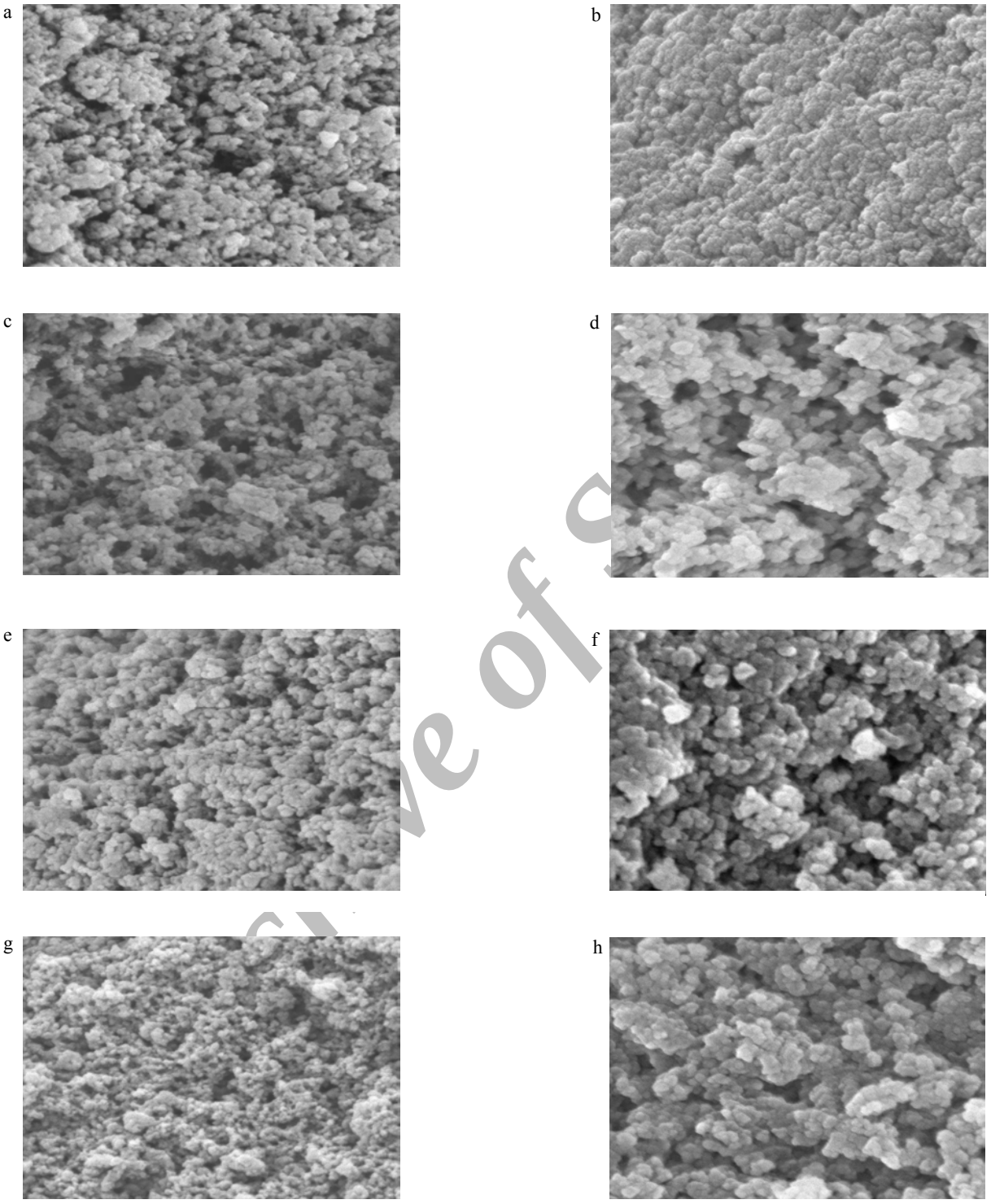

Fig. 4: Pressure and flow rate effects on the microstructure of immobilized lipase from Mucor miehei in the hydrolysis of canola oil in $SCCO_2$. a,b: untreated immobilized enzyme; c,d: 24.0 MPa pressure and 3.9 L/min CO_2 flow rate (measured at ambient conditions); e,f: 38.0 MPa pressure and 3.9 L/min CO_2 flow rate; g,h: 24.0 MPa pressure and 0.5 L/min CO_2 flow rate. A batch of 15.0 g canola sample and 1.0 g immobilized enzyme was used. Micrographs are reported at 30,000X magnification level. Left column: samples gold-sputtered; right column: samples chromium-sputtered.

level. If the presence of CO_2 at high pressure had a reversible effect (i.e., a temporary effect) on the enzyme, it would have disappeared by the depressurization of the solvent. However, if such effects are irreversible (i.e. permanent), then physical observation by SEM may be helpful depending on the level of structural changes. Whether the application of CO_2 at high pressures will have additional effects on a specific enzyme other than the effects related to the high pressure alone depends on the enzyme itself. When seven free amino acids were exposed to $SCCO_2$, there was no change in their structure except for glutamine, which partially reacted with CO_2 [23]. Furthermore, *L*-arginine displayed no changes over the pH range of 3-7 when it was exposed to $SCCO_2$ at 30 MPa and 80°C for 6 h [24]. However, at pH=9, ≥10% conversion to arginine bicarbonate was observed. In agreement with *Kamat et al.* [10,11], there might have been some reversible changes during the exposure time that could not have been detected by the methods used by *Weder et al.* [24]; i.e., thin-layer chromatography, ninhydrin reaction, etc. This was the case when *Kamat et al.* [10] using Laser Desorption Mass Spectrometry, LD/MS, showed that subtilisin was modified covalently during the exposure to high pressure CO_2 and that the covalent bond disappeared upon pressure release. In agreement with that, *Nakamura et al.* [25] observed a sharp peak in the adsorption isotherms of several proteins (such as casein, gelatin, gluten and ovalbumin) just above the critical pressure of CO_2 (8.3-8.8 MPa), which is very close to the pressure range reported by *Ikushima et al.* [13,26].

By monitoring the enzyme conformation using Fourier Transform Infrared, FTIR, spectroscopy in $SCCO_2$, *Ikushima et al.* [13] showed that over a limited range of pressure, 7.7-8.5 MPa, dramatic changes in *C. cylindracea* lipase occurred resulting in a higher reaction rate. The pressure range of esterification reactions where lipase from *C. cylindracea* catalyzed the production of optically active compounds overlapped with the pressure range in which sudden conformational changes took place [13]. When conducting lipase-catalyzed esterification of *n*-valeric acid and citronellol in $SCCO_2$, *Ikushima et al.* [26] observed a sharp increase in the reaction rate at pressures close to the critical region of CO_2. They speculated that the electron acceptability of $SCCO_2$ became appropriate at a given pressure to conduct a particular esterification reaction like that of *n*-valeric acid and citronellol. However, *Ishikawa et al.* [27] reported a slow decrease in the activity of glucoamylase and acid protease with a change in $SCCO_2$ density up to a certain point (0.82 and 0.60 g/cm^3, respectively), after which enzyme activity showed a sudden drop. Similar trends were observed with an increase in temperature or pressure. Although the results of *Ikushima et al.* [13,26] and *Ishikawa et al.* [27] do not appear to be in agreement, these results were obtained using different enzymes at different pressure ranges (*Candida cylindracea* lipase at 7-8 MPa for the former and glucoamylase at >15 MPa and acid protease at >8.5 MPa for the latter).

On the other hand, *Lozano et al.* [28] reported a positive effect due to pressure on the activity of α-chymotrypsin. Their studies on gas, liquid and super-critical media showed that the half-life of the enzyme increased proportionally with an increase in the density of CO_2 in any medium. They suggested that the increase in the density of a fluid could be used as a means of protecting enzymes from deactivation. *Lozano et al.* [28] related this important phenomenon to the increase in several parameters such as dielectric constant, polarity/ polarizability and/or Hildebrand solubility parameter of the solvent. These parameters have a direct effect on the hydrophilic property of the solvent resulting in enhanced enzyme stability by promoting solvent-protein interactions [28,29].

Effect of CO_2 flow rate

To study the effect of CO_2 flow rate on the enzyme efficiency, two separate enzyme batches (1.0 g each) were exposed to $SCCO_2$ at 24.0 MPa and 35°C at two different CO_2 flow rates of 0.5 and 3.9 L/min, measured at ambient conditions, over a 6-h reaction time (Fig. 5). Enzyme efficiency dropped at the beginning of the run with the higher CO_2 flow rate due to the oversupply of extracted oil onto the enzyme sites as well as drop in enzyme activity. Therefore, the additional TG supplied to the enzyme sites were not fully hydrolyzed. However, as the process continued, the supply of oil from the limited source of TG (in the extractor) onto the enzyme sites (in the reactor) were reduced and as a result enzyme was able to hydrolyze a higher fraction of the total TG received on the enzyme sites. Therefore, a higher enzyme efficiency was observed with the limited supply of oil towards the

end of the run. However, such pattern was not observed when applying the lower CO_2 flow rate. Extraction behavior at the lower CO_2 flow rate was proven to be controlled by an equilibrium throughout the 6-h run [4]. Consequently, there was a consistently equal supply of oil onto the enzyme sites throughout the entire run. The drop in the enzyme efficiency was also observed in this case due to the loss in enzyme activity/stability along the run. Such drop in enzyme efficiency was slow due to the lower level of TG supplied onto the enzyme sites when applying the lower CO_2 flow rate. At the higher CO_2 flow rate the linearity of the extraction curve and, therefore, the consistency of oil supply to the enzyme sites were maintained only for the first ~2 h [4] and thereafter the rate of oil supply was limited by the mass transfer rates of oil from the internal pores of canola flakes in the extraction process, which in turn influenced the reaction kinetics on the enzyme sites. Moreover, different CO_2 flow rates may induce different behaviors to the enzyme sites. At the higher CO_2 flow rates, the amount of water present on the enzyme sites is also different due to the higher stripping properties of CO_2. This by itself can be a reason for a sharper drop in the enzyme efficiency at the beginning of the run when applying the higher CO_2 flow rate. Nevertheless, despite all those differences between the enzyme environments in the two treatments, comparison of the micrographs from the treated enzymes with those of the untreated enzyme (Fig. 4c,d,g,h) did not reveal any sensible changes in the physical structure of the enzyme before and after exposure to CO_2 flow at the two levels applied in this study. Therefore, either it was not possible to characterize likely changes in the enzyme structure by SEM at this level of magnification (30,000X) or that the enzyme could withstand these conditions without going through any structural changes. Possible changes in enzyme clustering may also be deduced among the micrographs of enzymes with different treatments, which were beyond the expertise of the current authors.

Effect of enzyme load

To investigate the effect of enzyme load on the enzyme efficiency, a 1.0 and a 5.0 g enzyme batches were used in $SCCO_2$ at 24.0 MPa and 35°C. For each run, a batch of 15.0 g canola flakes was also loaded in the extraction chamber and a 0.5 L/min CO_2 flow rate was applied to the system. Enzyme efficiency was higher when using a higher level of enzyme load (Fig. 6), which is due to the extra catalytic sites provided to the reaction system. Because of a higher enzyme capacity, the drop in the enzyme efficiency as observed with the 1.0 g enzyme was not apparent with the 5.0 g enzyme batch during the time period applied in this study. Similar to the previous section, the comparison of the micrographs of the treated enzyme with those of the untreated enzyme did not indicate any obvious differences among the various batches of enzyme if mounted by carbon tape and sputter-coated with gold (Fig. 7a,c,e). However, when the samples were mounted using silver paint and chromium-sputtered (Fig. 7b,d,f), some grains from the 5.0 g batch (Fig. 7f) showed a substantial difference compared to those gold-sputtered. Such a difference in the structural appearance of the enzyme bead was probably due to a close contact among different enzyme grains leading to oil accumulation on the enzyme surfaces, which could not be carried away by CO_2 flow. Similar oil contamination was observed in the samples when they were evacuated from the reactor. The presence of residual oil in the 5.0 g enzyme bed was also evident when the amounts of the total oil extracted were compared: the amount of the oil collected was lower when using the 5.0 g enzyme in the bed. In the case of 5.0 g enzyme, the ratio of glass beads to enzyme was much less than that of the 1.0 g batch (2.7:5.0 vs. 7.9:1, respectively). Such modification was necessary due to the limited volume of the reactor cell. The difference in the water level on the enzyme sites for the 5.0 g batch may also have been a factor since enzymes originally possess certain amount of water on them and also water consumption and the stripping effects of the supercritical fluid were different.

Effect of canola load (oil content)

Effect of oil content on enzyme efficiency in the lipase-catalyzed hydrolysis of canola oil was investigated using two different levels of cooked canola flakes (3.0 and 15.0 g, loaded in the extraction chamber) at 24.0 MPa and 35°C. CO_2 flow rate through the system was 0.5 L/min for both runs. The enzyme efficiency was higher with the lower level of canola load (Fig. 8). When a higher level of canola load was used, a consistently higher level of oil supply was maintained onto the enzyme bed and therefore the total amount of oil

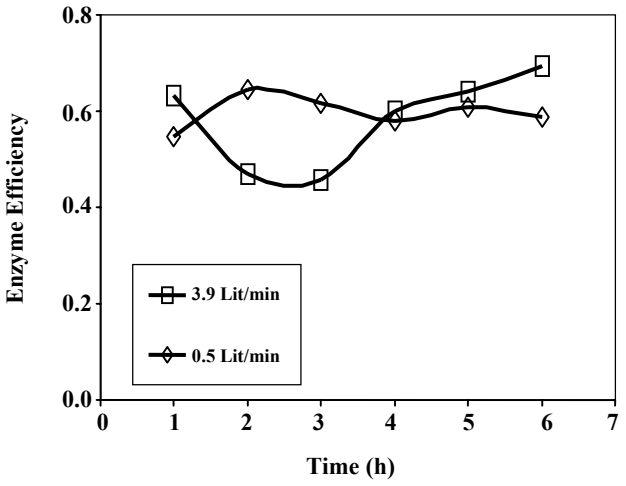

Fig. 5: Effect of CO_2 flow rate (0.5 and 3.9 L/min) on enzyme efficiency in the on-line extraction-reaction of canola oil at 24.0 MPa and 35°C using Lypozyme IM (immobilized lipase from Mucor miehie). Canola load 15.0 g and enzyme load 1.0 g.

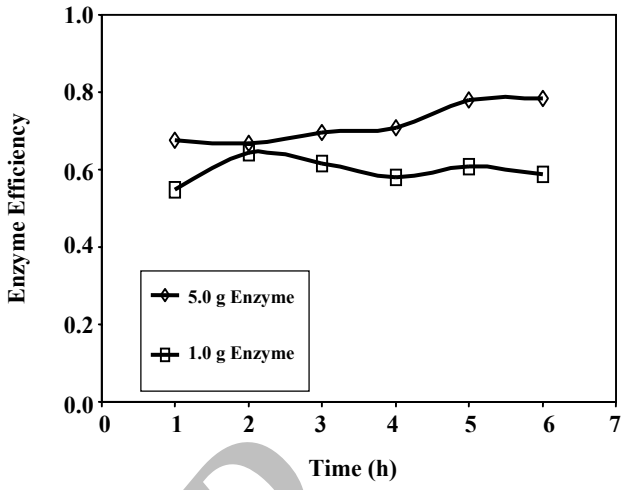

Fig. 6: Effect of enzyme load (1.0 and 5.0 g) on enzyme efficiency in the on-line extraction-reaction of canola oil at 24.0 MPa and 35°C using Lypozyme IM (immobilized lipase from Mucor miehie). CO_2 flow rate 0.5 L/min (measured at ambient conditions), canola load 15.0 g.

contacting the enzyme bed was also higher (0.977 g vs. 0.725 g, respectively). In addition, a higher amount of water was received by the enzyme when using a higher level of canola load since more water was extracted from the 15.0 g canola batch than from the 3.0 g batch due to the higher level of water available in the 15.0 g oilseed. Furthermore, with the depletion of oil from the 3.0 g canola batch, enzyme can hydrolyze a higher fraction of the supplied TG and as a result enzyme efficiency increased towards the end of the run. Such differences in the amount of oil and water introduced to the enzyme bed resulted in the differences observed in the enzyme efficiency as shown in Fig. 8. However, micrographs of the enzyme undergone these conditions and for the 6-h period of this study did not show any apparent difference (s) compared to those of an untreated enzyme except for possible clustering changes as discussed earlier (Fig. 7g,h).

CONCLUSIONS

Enzyme efficiency in the lipase-catalyzed hydrolysis of canola oil in $SCCO_2$ was influenced by a change in pressure, enzyme load, CO_2 flow rate and oil content of $SCCO_2$. An increased level of enzyme load and decreased level of oil content resulted in a higher enzyme efficiency but a higher CO_2 flow rate resulted in a complex behavior of enzyme resulting in different enzyme efficiencies at different times. On the other hand, the micrographs of the applied enzyme in this study obtained by SEM at 30,000X magnification was not able to clearly demonstrate any possible changes that may have occurred in the enzyme microstructure and therefore, a different strategy (such as a higher magnification level) and/or the application of other instrumental approaches such as Infrared (IR) or Mass Spectrometry (MS) should be sought. This study revealed that if no destructive factor, such as an excessive amount of water, were imposed, the enzyme was able to maintain its stability allowing its application in continuous reactors operating under supercritical conditions for many hours without a need to refresh the enzyme bed. In this study, it was not possible to consider the use of the free (unsupported) enzyme (i.e. the enzyme not immobilized) nor the support material alone (i.e. with no enzyme) to identify possible function(s) of the enzyme support on the enzyme stability or structure as sought in this study.

Acknowledgements

Gratitude is expressed to the Natural Sciences and Engineering Research Council of Canada for financial support, Novo Nordisk for providing enzyme and CanAmera foods for providing canola samples. K. Rezaei thanks the Ministry of Culture and Higher Education of Iran for financial assistance. Also, the assistance provided by George D. Braybrook in obtaining the Scanning Electron Microscope images is gratefully appreciated.

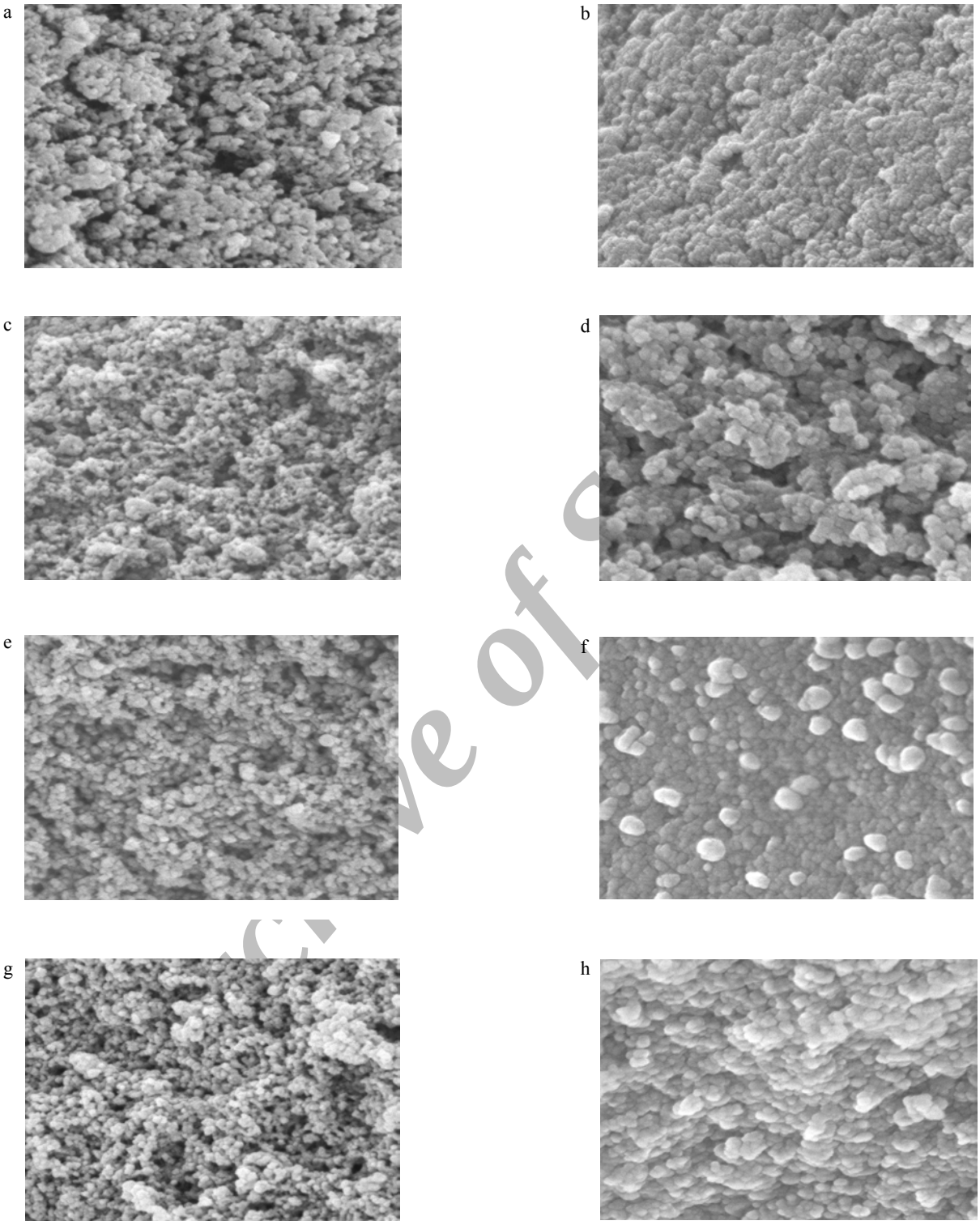

Fig. 7: Effects of enzyme and canola loads on the microstructure of immobilized lipase from Mucor miehei in the hydrolysis of canola oil in $SCCO_2$ at 24.0 MPa and 35°C and 0.5 L/min CO_2 flow rate (measured at ambient conditions). a,b: untreated immobilized enzyme; c,d: 1.0 g enzyme and 15.0 g canola flakes; e,f: 5.0 g enzyme and 15.0 g canola flakes; g,h: 1.0 g enzyme and 3.0 g canola flakes. Micrographs are reported at 30,000X magnification level. Left column: samples gold-sputtered; right column: samples chromium-sputtered.

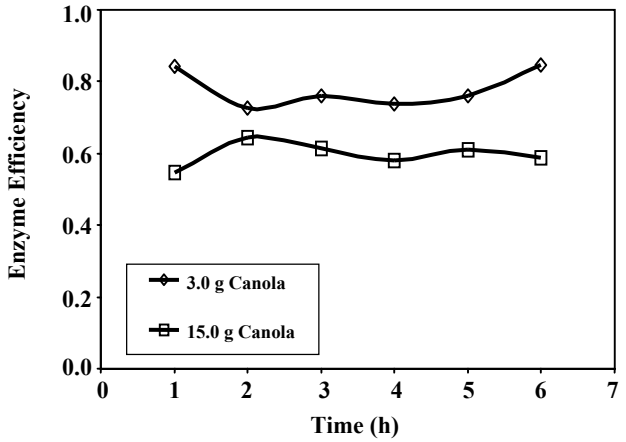

Fig. 8: Effect of canola load (3.0 and 15.0 g) on enzyme efficiency in the on-line extraction-reaction of canola oil at 24.0 MPa and 35°C using Lypozyme IM (immobilized lipase from Mucor miehie). CO_2 flow rate 0.5 L/min (measured at ambient conditions), enzyme load 1.0 g.

Received : 1st August 2005 ; Accepted : 24th April 2006

REFERENCES

[1] Yu, Z. R., Rizvi, S. S. H. and Zollweg, J. A., Enzymatic esterification of fatty acid mixtures from milk fat and anhydrous milk fat with canola oil in supercritical carbon dioxide, *Biotech. Prog.,* **8**, 508 (1992).

[2] Klemann, L. P., Aji, K., Chrysam, M. M., D'amelia, R. P., Henderson, J. M., Huang, A. S., Otterburn, M. S. and Yarger, R. G., Random nature of triacylglycerols produced by the catalyzed interest-erification of short- and long-chain fatty acid triglycerides, *J. Agric. Food Chem.,* **42**, 442 (1994).

[3] Saito, S., Research activities on supercritical fluid science and technology in Japan - A review, *J. Supercrit. Fluids*, **8**, 177 (1995).

[4] Rezaei, K. and Temelli, F., On-line extraction-reaction of canola oil using immobilized lipase in supercritical CO_2, *J. Supercrit. Fluids*, **19**, 263. (2001).

[5] Cao, X. and Ito, Y., Supercritical fluid extraction of grape seed oil and subsequent separation of free fatty acids by high-speed counter-current chromato-graphy, *J. Chromat. A*, **1021**, 117 (2003).

[6] Nagesha, G. K., Manohar, B. and Sankar, K. U., Enzymatic esterification of free fatty acids of hydrolyzed soy deodorizer distillate in supercritical carbon dioxide, *J. Supercrit. Fluids*, **32**, 137 (2004).

[7] Letourneau, J. J., Vigneau S., Gonus P. and Fages J., Micronized cocoa butter particles produced by a supercritical process, *Chem. Eng. Proc.*, **44**, 201 (2005).

[8] Rezaei, K. and Temelli, F. Lipase catalyzed hydrolysis of canola oil in supercritical CO_2, *J. Am. Oil Chem. Soc.*, **77**, 903 (2000).

[9] Marty, A., Chulalaksananukul, W. and Condoret, J. S., Kinetics of lipase-catalyzed esterification in supercritical CO_2, *Biotech. Bioeng.,* **39**, 273 (1992).

[10] Kamat, S., Critchley, G., Beckman, E. J. and Russell, A. J., Biocatalytic synthesis of acrylates in organic solvents and supercritical fluids . 3. Does carbon dioxide covalently modify enzymes?, *Biotech. Bioeng.*, **46**, 610 (1995).

[11] Kamat, S., Barrera, J., Beckman, E. J. and Russell, A. J., Biocatalytic synthesis of acrylates in organic solvents and supercritical fluids, I. Optimization of enzyme environment, *Biotech. Bioeng.*, **40**, 158 (1992).

[12] Lee, H. S., Lee, W. G. and Park, S. W., Starch hydrolysis using enzyme in supercritical carbon dioxide, *Biotech. Techniq.,* **7**, 267 (1993).

[13] Ikushima, Y., Saito, N., Arai, M. and Blanch, H. W., Activation of a lipase triggered by interactions with supercritical carbon dioxide in the near-critical region, *J. Phys. Chem.,* **99**, 8941 (1995).

[14] Randolph, T. W., Blanch, H. W. and Clark, D. S., "Biocatalysis in supercritical fluids. In Biocatalysis for Industry", J. S. Dordick (Ed.), pp. 219-237 Plenum Press, New York. (1991).

[15] Vasudevan, P. T. and Weiland, R., H., Studies on the morphology of immobilized catalase, *Chem. Eng. J.*, **55**, B41 (1994).

[16] Yu, T., Liu, H. Deng, J. and Liu, Y., Characterization of regenerated silk fibroin membrane for immobilizing glucose oxidase and construction of a tetrathiafulvalene-mediating glucose sensor, *J. Appl. Polym. Sci.,* **58**, 973 (1995).

[17] Liu, Y., Liu, H., Qian, J., Deng, J. and Yu, T., Immo-bilization of glucose oxidase in the regenerated silk fibroin membrane, Characterization of the membrane structure and its application to an amperometric glucose sensor employing ferrocene as electron shuttle, *J. Chem. Technol. Biotech.*, **64**, 269 (1995).

[18] Liu, H., Zhang, X., Zhang, Z., Qi, D., Fan, Y., Liu,

Y., Liu, S. and Yu, T., Characterization of composite membrane of poly (vinyl alcohol) and regenerated silk fibroin for immobilization of horseradish peroxidase and amperometric neckelocene-mediated sensor sensitive to hydrogen peroxide, *J. Chem. Technol. Biotech.*, **67**, 77 (1996).

[19] Verghese, M. M., Ramanathan, K., Ashraf, S. M. and Malhotra, B. D., Enhanced loading of glucose oxidase on polyaniline films based on anion exchange, *J. Appl. Polym. Sci.*, **70**, 1447 (1998).

[20] Miller, D. A., Blanch, H. W. and Prausnitz, J. M., Enzyme-catalyzed interesterification of triglycerides in supercritical carbon dioxide, *Ind. Eng. Chem. Res.*, **30**, 939 (1991).

[21] Balaban, M. O., Arreola, A. G., Marshall, M. and Cornell, J., Inactivation of pectinesterase in orange juice by supercritical carbon dioxide, *J. Food Sci.,* **56**, 743 (1991).

[22] Owusu-Yaw, J., Marshall, M. R., Koburger, J. A. and Wei, C. I., Low pH inactivation of pectin-esterase in single strength orange juice, *J. Food Sci.*, **53**, 504 (1988).

[23] Weder, J. K. P., Studies on proteins and amino acids exposed to supercritical carbon dioxide extraction conditions, *Food Chem.*, **15**, 175 (1984).

[24] Weder, J. K. P., Boker, M. V. and Hegarty, M. P., Effect of supercritical carbon dioxide on arginine, *Food Chem.,* **44**, 287 (1992).

[25] Nakamura, K., Hoshino, T. and Ariyama, H., Adsorption of carbon dioxide on proteins in the supercritical region, *Agric. Biol. Chem.,* **55**, 2341 (1991).

[26] Ikushima, Y., Saito, N., Hatakeda, K. and Sato, O., Promotion of a lipase-catalyzed esterification in supercritical carbon dioxide in near-critical region, *Chem. Eng. Sci.*, **51**, 2817 (1996).

[27] Ishikawa, H., Shimoda, M., Kawano, T. and Osajima, Y., Inactivation of enzymes in an aqueous solution by micro-bubbles of supercritical carbon dioxide, *Biosci. Biotech. Biochem.*, **59**, 628 (1995).

[28] Lozano, P., Avellaneda, A., Pascual, R. and Iborra, J. L., Stability of immobilized alpha-chymotrypsin in supercritical carbon dioxide, *Biotech. Lett.,* **18**, 1345 (1996).

[29] Vermuë, M. H., Tramper, J., de Jong, J. P. L. and Oostrom, W. H. M., Enzymic transesterification in near-critical carbon dioxide. Effect of pressure, Hildebrand solubility parameter and water content, *Enz. Microb. Technol.*, **14**, 649 (1992).