Journal of Molecular Catalysis B: Enzymatic 77 (2012) 92–97

Contents lists available at SciVerse ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Evaluation of diatomaceous earth supported lipase sol–gels as a medium for enzymatic transesterification of biodiesel

S.M. Meunier*, R.L. Legge

Department of Chemical Engineering, University of Waterloo, 200 University Avenue West, Waterloo, Ontario, Canada N2L 3G1

ARTICLE INFO

Article history:
Received 26 October 2011
Received in revised form 12 January 2012
Accepted 15 January 2012
Available online 25 January 2012

Keywords:
Enzyme immobilization
Biodiesel
Sol–gel
Transesterification

ABSTRACT

Immobilized lipase has the potential to be the catalyst of choice for biodiesel production since it is efficient, effective, and environmentally friendly; however, the stability and activity of lipase must be addressed before enzymatic biodiesel production processes can be industrially accepted. This study investigates an enzyme immobilization procedure that immobilizes lipase in a sol–gel supported on diatomaceous earth (Celite® R632), and determines its potential for biodiesel production in terms of achievable conversion and apparent stability. Four immobilized materials (lipase sol–gels with and without Celite® at two protein loading levels) were compared in terms of their immobilized protein content, conversion of methanol to methyl oleate, lipase activity, long term stability, and glycerol–water adsorption. The Celite® R632 sol–gel with high protein loading achieved the maximum conversion in the 6-h reaction period (90%). A drying step was found to be advantageous prior to the reaction, and the absorption of glycerol–water on the Celite® was only found to be significant at high levels of glycerol. The material was found to be very stable upon storage at 4 °C for up to 1.5 years, losing only about 15% of its percent conversion capacity per year. Based on this study, the supported immobilization technique shows significant potential as a novel catalyst for biodiesel production.

1. Introduction

Lipase is a triacylglycerol hydrolase and is responsible for the hydrolysis of ester bonds in triglycerides. At hydrophobic interfaces, lipases are known to undergo interfacial activation which causes a surge in enzymatic activity [1]. Enzymatic reactions typically have many advantages such as enhanced specificity and efficiency, however, decreased activity and stability are common elements of concern when working with enzymes.

One way to extend the operational stability, and thus decrease the effective cost of the enzyme, is by immobilization. Although biodiesel production with alkali catalysts is known to be the least costly option, processes using immobilized lipase are significantly less costly than those using free lipase [2]. Jegannathan et al. also show that if immobilized lipase is reused more than five times or the production cost of the lipase is reduced significantly, enzymatic biodiesel production could be competitive with the alkali biodiesel process [2].

Enzyme entrapment in polymers such as sol–gels provides a stable enzyme support with very strong bonds while not requiring complex chemical preparation [3]. Both the thermal and chemical stability and activity of enzymes can be improved via sol–gel immobilization [3–5]. Further, ω-transaminases encapsulated via a supported immobilization procedure with Celite® sol–gels exhibit enhanced activity even at extreme conditions such as high pH and temperature, and can be recycled up to eight times without dramatically reducing the achievable conversion [6]. Other studies comparing Celite® sol–gels show similar results using lipase as the enzyme of interest [7,8].

Due to environmental and economical concerns, biodiesel has become an invaluable alternative to standard diesel fuels [9]. Biodiesel is comprised of fatty acid alkyl esters and is produced via the transesterification of oils. Conventionally, biodiesel is produced using either an alkali or acid catalyst, but using lipase as the catalyst for transesterification could provide significant advantages over these traditional processes. Enzymatic processes self-adapt to changes in raw material quality, produce biodiesel in few processing steps, use little energy, produce little wastewater, and yield high quality by-products [10]. The challenges associated with enzymatic transesterification include low reaction rates, high enzyme cost, and the potential for enzyme deactivation [11].

Since methanol, glycerol and water are all known to have inhibitive effects on lipase each of these components must be closely monitored in the biodiesel production process. Methanol is a substrate as well as a lipase inhibitor in the biodiesel production process; therefore, lower than stoichiometric ratios of methanol are commonly used for biodiesel production to avoid enzyme deactivation [12–16]. Glycerol is a by-product of enzymatic biodiesel

* Corresponding author. Tel.: +1 519 888 4567x31648; fax: +1 519 888 4347.
E-mail address: sarah.meunier@uwaterloo.ca (S.M. Meunier).

1381-1177/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2012.01.014

Table 1
Description of the lipase preparations used for analysis in terms of the support material, sol–gel formulation, and lipase solution concentration.

Lipase preparation	Support material	Sol–gel	Lipase solution
C-SG-4	Celite® R632	80% PTMS/20% TMOS	4 mg/mL
C-SG-12	Celite® R632	80% PTMS 20% TMOS	12 mg/mL
U-SG-4	Unsupported	80% PTMS/20% TMOS	4 mg/mL
U-SG-12	Unsupported	80% PTMS/20% TMOS	12 mg/mL
Free	Unsupported	No sol–gel	4 mg/mL

production that is inhibitory to the lipase, but glycerol produced during transesterification can be absorbed by silica beds [17–19]. In terms of water absorption, according the Celite® supplier, World Minerals, the water absorption capacity of Celite® is up to 500% by weight, depending on the type of Celite® considered. This is a crucial parameter since, although water is necessary for lipase activation in biodiesel production, excess water levels will inhibit the lipase by occupying the enzyme support pore space and limiting contact between the enzyme and substrates [9].

This study considers the enzymatic activity of lipase immobilized in Celite® R632 sol–gels based on a supported immobilization scheme. A comparison is made between the achievable enzymatic conversion of methanol to methyl oleate for unsupported sol–gels and sol–gels supported on Celite® R632 at both high and low protein content levels. Further, the glycerol–water absorption is considered for both Celite® R632 and sol–gel Celite® R632 using thermogravimetric analysis in an attempt to elucidate the possible effects of glycerol and water in a biodiesel process for lipase immobilized in Celite® R632 sol–gels. This information is valuable in evaluating the potential of Celite® R632 sol–gels as a supported immobilization medium for enzymatic biodiesel production.

2. Experimental

2.1. Materials

Celite® samples were a gift from World Minerals (Santa Barbara, CA). Lipase (NS44035) was a gift from Novozymes North America Inc. (Franklinton, NC). The biological source of NS44035 cannot be disclosed by the supplier; the activity of NS44035 is 20,000 PLU/g. Tetramethyl orthosilicate (TMOS), trimethoxypropylsilane (PTMS), triolein, glycerol, methyl oleate and methyl heptadecanoate (HDA-ME) were obtained from Sigma–Aldrich Canada Ltd. (Oakville, ON). Acetonitrile was obtained from EMD Chemicals (Gibbstown, NJ). Sodium phosphate was obtained from Mallinckrodt Baker (Phillipsburg, NJ). Hexane and hydrochloric acid were obtained from Fisher Scientific Company (Ottawa, ON). Ultrapure water was produced using a Milli-Q water purification system from Millipore (Billerica, MA). All other chemicals were obtained from local suppliers.

2.2. Methods

2.2.1. Immobilization of lipase

Four different lipase sol–gels were produced for analysis—with and without Celite® R632 as a support material and with high and low concentrations of lipase (Table 1).

To immobilize lipase in diatomaceous earth sol–gels, 0.08 mol PTMS and 0.02 mol TMOS were hydrolyzed in the presence of 0.1 mol ultrapure water and 200 μL HCl (0.1 M). The mixture was sonicated for 1 h to allow for complete hydrolization of the precursors. The precursor solution was then rotary evaporated in a heated water bath at 40 °C for 30 min to remove excess water and alcohol. A solution of lipase and phosphate buffer (50 mM, pH 7.0) with an approximate protein concentration of 4 mg/mL was prepared, and 14 mL was added to the hydrolyzed precursor solution. For the high lipase concentration Celite sol–gels, the lipase solution was used undiluted at a concentration of approximately 12 mg/mL. The resultant mixture was added to the support material with approximately 3 mL sol–gel mixture for every 2 g of Celite® R632. After thorough mixing, the sol–gel Celite was deposited in a Petri dish, sealed and aged at 4 °C for 24 h. The Celite® sol–gel was then dried uncovered at 4 °C until the drying rate was less than 1 mg/h. Finally, the supported gel was removed from the Petri dish and washed twice with phosphate buffer (50 mM, pH 7.0, 5 mL buffer per gram of sol–gel for each wash) to remove any protein that was not completely immobilized within the gel. Excess solvent was evaporated from the gel at room temperature overnight prior to storing the gels in a sealed container at 4 °C.

Unsupported gels were prepared in a similar manner except that the evaporated precursor and enzyme mixture was deposited directly into the Petri dish for aging and drying, the dried sol–gel was crushed in a mortar upon removal from the Petri dish, and the washing solutions were separated from the sol–gel by centrifugation.

2.2.2. Protein measurement

The total protein content of the gels was calculated using a mass balance of the protein content of the enzyme solution loaded to the sol–gels and content in the two buffer wash solutions. The degree of immobilization was calculated as the percentage of protein in the sol–gel compared to the amount of protein desired in the supported sol–gel. The amount of protein was quantified using a Varian HPLC system (Varian Inc., Mississauga, ON) equipped with an Agilent Zorbax Bio Series GF-250 column (Agilent Technologies, Mississauga, ON) and calibrated using a BCA protein assay kit (Pierce Biotechnology Inc., Rockford, IL). The mobile phase for the HPLC analysis was 200 mM phosphate buffer (pH 7.0) and detection at a wavelength of 280 nm.

2.2.3. Enzymatic lipid transesterification

The enzymatic activity of the supported lipase sol–gels was determined by GC–MS analysis. The reactions were carried out at 40 °C with agitation for 6 h. The reaction mixture consisted initially of approximately 1 g of the supported lipase sol–gel, 4 mmol of triolein and 4 mmol of methanol (total reaction volume 3.89 mL). Each hour, a 10 μL sample was removed from the reaction vial and diluted in 990 μL hexane with 100 μL of the internal standard, HDA-ME. The formation of methyl oleate, the reaction product, was followed using a Varian GC–MS system (CP-3800 gas chromatograph, Saturn 2000 mass spectrometer/mass spectrometer) equipped with a CP-Wax 52 CB fused silica column (CP8513, Varian Inc., Mississauga, ON). 1 μL samples of the diluted reaction mixture were injected into the GC at an injector temperature of 250 °C and a split ratio of 50. Helium was used as the carrier gas with a column flow of 1 mL/min. The GC oven temperature was initially set to 170 °C for 10 min, ramped at 10 °C/min to 250 °C, and held at 250 °C for 2 min.

The enzymatic activity of the dried sol–gel formulations were carried out in the same manner as the original gels with the exception that prior to the enzymatic assay the gels were dried overnight in a 60 °C oven. At this point, no further change in mass was observed from the drying process and thus any remaining water was assumed to be completely removed from the sol–gel formulation.

Using the GC–MS method described, a calibration was completed based on a methyl oleate standard (40–800 mM) and used as the basis for all methyl oleate concentrations provided. From the GC–MS chromatograms, methyl oleate was the only visible peak indicating that no side reactions occurred. The enzymatic activity of the lipase was determined from the slope of the methyl oleate

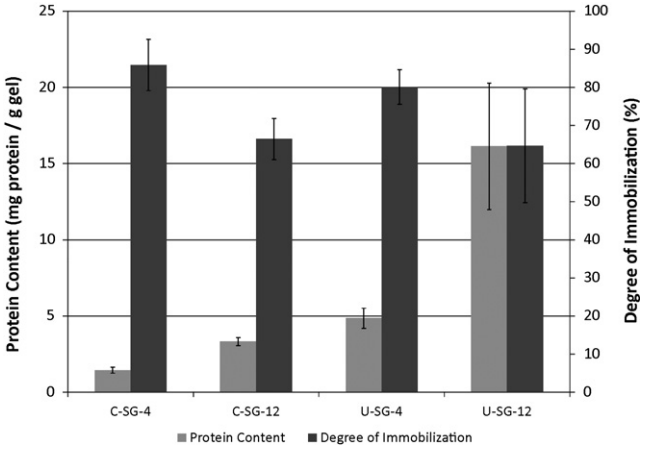

Fig. 1. Protein content and degree of protein immobilization for four sol–gel formulations. Error bars represent a 95% confidence interval.

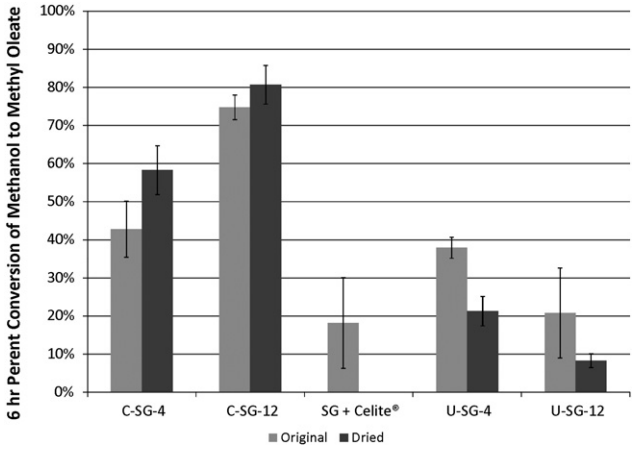

Fig. 2. Percent conversion of methanol to methyl oleate based on a 6-h batch reaction for each sol–gel formulation without and with a drying step prior to the enzymatic assay. Error bars represent a 95% confidence interval.

concentration–time profile over the weight of immobilized enzyme material used.

2.2.4. Desorption of glycerol and water

The desorption of glycerol and water from Celite® R632 upon equilibration was measured using a thermogravimetric analyzer (Q500 TGA, TA Instruments, New Castle, DE). Prior to analysis, each sample was immersed in the desired solution containing glycerol and water at various concentrations (0%, 10%, 25%, 50%, 75% and 100% by volume glycerol) and equilibrated for 24 h. The sample was then washed with water to remove excess glycerol and air dried overnight. The TGA analysis method ramped the temperature from 30 °C to 400 °C at 10 °C/min followed by air cooling for 10 min under 50 mL/min N_2 gas. The onset of desorption (temperature at which 0.025% mass loss is achieved), the peak desorption rate (maximum mass loss rate achieved), the peak temperature (temperature at the peak desorption rate), and the total mass loss (percent mass lost at the end of the analysis) were determined from the weight loss curve and the derivative weight loss curve.

3. Results and discussion

3.1. Physical properties

Comparing the protein content and the degree of immobilization of the four different lipase sol–gels formed, several phenomena were observed (Fig. 1). First, for both the low and high protein level sol–gels, the unsupported sol–gels have higher protein contents in comparison to the Celite® supported sol–gels. Since there is additional material added to the Celite® sol–gels, lower protein content per gram is expected for these gels.

The protein level immobilized in the sol–gel was not proportional to target load for the sol–gel during the formation procedure (Fig. 1). For the Celite® sol–gels the protein content was approximately double when triple the lipase was loaded, but for the unsupported sol–gels the protein content was approximately triple as expected. One possible explanation for the lack of proportionality for the Celite® sol–gels is that the excess of lipase in the triple unsupported formulation caused protein aggregation and subsequent immobilization. This effect might not be as pronounced for the lower levels of lipase because there is not enough lipase in the formulation and for the triple lipase Celite® system because the presence of Celite® reduces the apparent protein concentration.

The observed degree of protein immobilization for the Celite® sol–gels is comparable to that for the unsupported sol–gels (Fig. 1). When the amount of lipase is tripled, the degree of immobilization decreases slightly for both the Celite® and unsupported sol–gels. Since there is excess lipase in triple lipase sol–gels, there is more opportunity for lipase that is incompletely entrapped to be easily removed during the washing steps.

The high protein content unsupported sol–gel (U-SG-12) did exhibit much more variability than the other immobilization regimes (Fig. 1). This is likely caused by the potential for aggregation, incomplete immobilization and lipase deactivation during the sol–gel formation procedure. With such high levels of protein and without the Celite® support material, the lipase is much more exposed and thus more sensitive to environmental changes. This enhanced potential for enzyme deactivation and poor immobilization can conceivably greatly reduce the reproducibility of the lipase sol–gel immobilization procedure.

3.2. Enzymatic properties

Free lipase at a comparable level to that found in the immobilized lipase preparation C-SG-4 was assayed to determine a base methyl oleate conversion level for comparison to the immobilized preparations. Based on GC–MS analysis, no measurable methyl oleate was detected during the 6 h reaction.

Based on the percent conversion after 6 h for methyl oleate production (Fig. 2), the Celite® R632 sol–gels exhibited an increased conversion when the amount of lipase loaded onto the gels was tripled. In addition, the Celite® supported sol–gels achieved slightly more conversion when dried due to an excess of water absorbed on the support material since Celite® is absorbent and lipase is inhibited by excess water content.

The unsupported sol–gels demonstrate the opposite trend—the sol–gel preparation with triple lipase achieves a lower percent conversion than the regular lipase loading and the dried gel preparations have lower conversion than those that are not dried. As discussed with regards to the protein content of the gels, this formulation does have much more variability than the other formulations which could be caused by aggregation, incomplete immobilization, and deactivation of the protein due to the excess quantities of protein. Since there is no Celite® present in the unsupported sol–gels to remove the water, drying the enzyme preparation may result in inactivation of the lipase rather than removing the excess water and thereby causing the reduction in product concentration.

In comparison to the Celite® supported sol–gel, adding neat Celite® to the unsupported sol–gel (SG + Celite®) greatly reduces

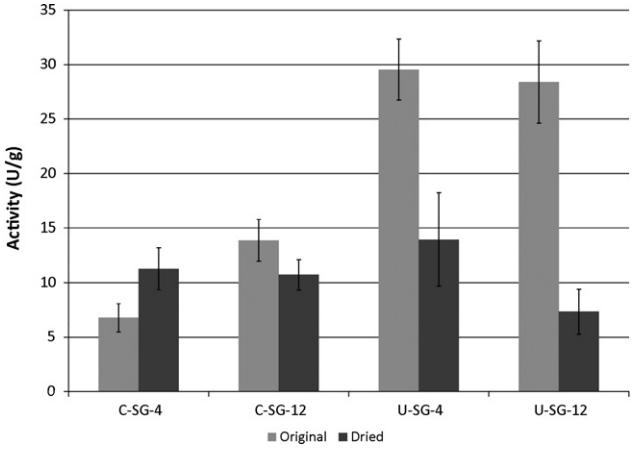

Fig. 3. The enzymatic activity on a per gram of material basis for each sol–gel formulation as determined from the conversion of methyl oleate and the protein content of the gels. Both the sol–gel formulations without and with a drying step prior to the reaction were considered. Errors bars represent a 95% confidence interval.

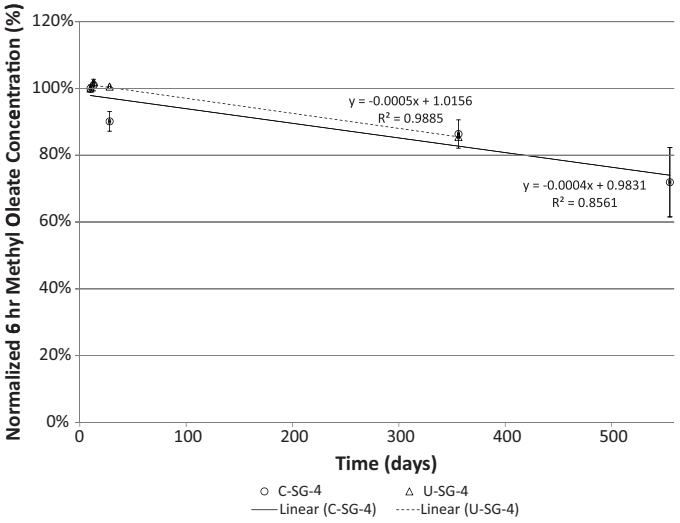

Fig. 4. Average percent conversion of methanol to methyl oleate for sol–gels after storage at 4 °C for unsupported (△) and Celite® R632 supported sol–gels (◯). The lines represent the lines of best fit for the unsupported sol–gel (broken) and the Celite® R632 supported sol–gel (solid). Error bars represent a 95% confidence interval.

the percent conversion likely due to reactant, product and water absorption by the Celite® thereby preventing the reaction from progressing. Comparing the supported and unsupported sol–gel formulations, the addition of the Celite® as a support material for the sol–gel increases the percent conversion to methyl oleate for both sol–gel formulations.

Comparing the activity of the different sol–gel preparations on a mass basis (Fig. 3) reveals that the dried unsupported and supported sol–gels all have comparable activities. This demonstrates the beneficial effects of using Celite® and providing a support since the Celite® sol–gels contain much less enzyme per gram of material in comparison to the unsupported sol–gels (Fig. 1). The unsupported sol–gel preparation with the higher level of lipase (U-SG-12) does have a slightly lower activity in comparison with the other materials when dried which is likely due to the excess lipase in the preparation which is either inactive and/or inaccessible to the substrates.

The unsupported sol–gels have a much higher activity than the Celite® sol–gels when assayed without a drying step. The adsorptive properties of the Celite® and the inhibition of lipase caused by excess water are the expected causes of this phenomenon. Despite the increased lipase content (Fig. 1) in the high concentration lipase unsupported sol–gel (U-SG-12), the activity is comparable to the regular lipase level unsupported sol–gel (U-SG-4). Therefore, the excess lipase in this system may not be accessible to the substrates or has been rendered inactive.

The Celite® supported sol–gels and the unsupported sol–gels were also compared based on their stability over a period of approximately 1.5 years (Fig. 4). The unsupported and Celite® supported sol–gel formulations show almost identical trends with a very gradual decrease in product output over time. The unsupported sol–gel (U-SG-4) has a 0.05% decrease in product concentration per day while the Celite® supported sol–gel (C-SG-4) has a 0.04% decrease per day. This is about an 18% (U-SG-4) and 15% (C-SG-4) decrease in product concentration per year. This indicates that a very stable enzyme formulation has been developed with a long shelf life when stored at 4 °C.

3.3. Absorptive properties

A TGA spectrum (Fig. 5) shows typical weight-time and derivative weight-time profiles for a sample of Celite® R632 sol–gel (C-SG-4). The peak desorption and total mass loss parameters are indicated on the graph. These profiles are used to determine the onset of desorption, peak desorption rate, peak temperature, and total mass loss.

Based on the weight change with respect to temperature data obtained from the TGA analysis, the temperature at which desorption was established for each case (10%, 25%, 50%, 75%, and 100% glycerol for both neat Celite® and the Celite® sol–gel C-SG-4, data not shown) was compared. The average onset of desorption for all cases was 113.3 ± 1.2 °C. This indicates that the observed desorption is caused by a combination of the water and the glycerol rather than the components separately.

The peak desorption rate obtained from the TGA analysis is a measure of the level of adhesion of solvent to the Celite® or Celite® sol–gel with respect to the cohesion of solvent molecules to each other. High peak desorption rates indicate that the solvent coheres more strongly to itself and low peak desorption rates signify that the solvent molecules adhere very well to the support material. The total mass loss indicates the absorptive capacity of the Celite® or Celite® sol–gel with respect to the applicable glycerol–water

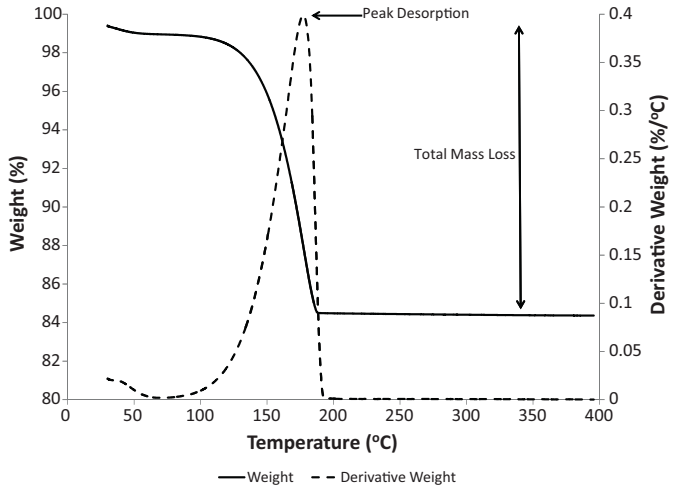

Fig. 5. Typical TGA profile including the sample weight (solid) and the derivative weight (broken). The peak desorption point and the total mass lost are indicated on the graph. The sample shown is Celite® R632 sol–gel (C-SG-4) at 50% glycerol equilibrating solution.

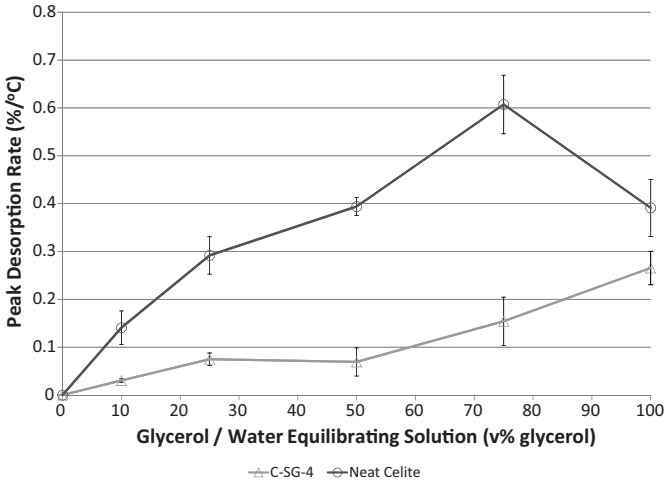

Fig. 6. Peak desorption rates for Celite® R632 sol–gels (△) and plain Celite® R632 (○) based on different levels of glycerol in the equilibrating solution. Error bars represent a 95% confidence interval.

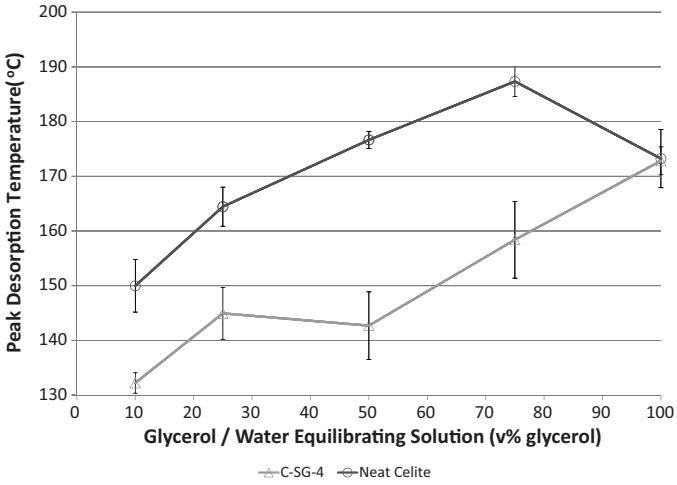

Fig. 8. Peak desorption temperatures for Celite® R632 (△) sol–gels and plain Celite® R632 (○) based on different levels of glycerol in the equilibrating solution. Error bars represent a 95% confidence interval.

solution. At the end of the TGA analysis, any glycerol and water absorbed and retained by the material are subsequently desorbed and thus quantified by the total mass loss.

Considering both the peak desorption rate (Fig. 6) and the total mass loss (Fig. 7) when neat Celite® and Celite® sol–gels are incubated in glycerol–water solutions similar trends are evident. Both the peak desorption rates and the total mass loss are higher in each case for the neat Celite® than for the Celite® sol–gel. It is likely that the sol–gel provides a protective barrier that reduces absorption of the glycerol–water solution.

Additionally, the amount of solution absorbed does generally increase with increasing percentage of glycerol in the equilibrating solution. One notable exception is the decrease for the neat Celite® from 75% glycerol to 100% glycerol. This shows that the water is necessary for absorption of glycerol onto the Celite. However, this is not the case for the Celite® sol–gel. The increasing adsorption trend of the supported sol–gel material is not affected by eliminating the water from the equilibrating solution.

When the equilibrating solution consisted solely of water, there was no evidence of desorption from the neat Celite® or sol–gel Celite® (Figs. 6 and 7). This is evidence that the water is not readily retained by the Celite® material despite its absorptive properties.

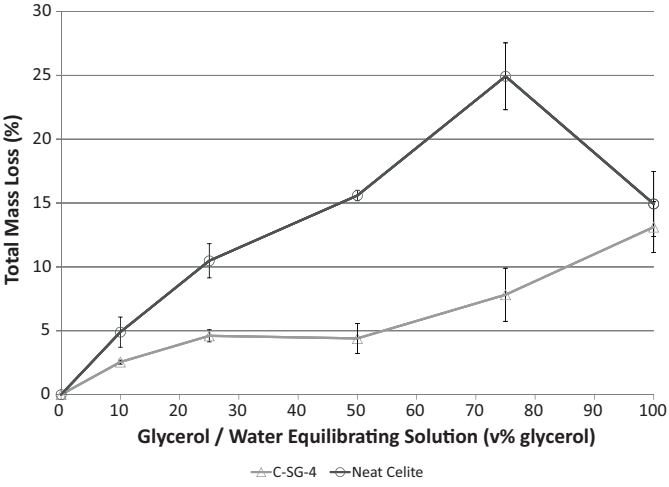

Fig. 7. Total percentage mass loss for Celite® R632 sol–gels (△) and plain Celite® R632 (○) based on different levels of glycerol in the equilibrating solution. Error bars represent a 95% confidence interval.

The peak desorption temperature as determined by the TGA is a measure of the strength of cohesion of the glycerol–water solution to the Celite® or Celite® sol–gel material. The peak desorption temperatures for each solution were different (Fig. 8), ranging from 132 °C to 187 °C, and showed similar trends to those observed for the peak desorption rate (Fig. 6) and the total mass loss (Fig. 7). One phenomena observed is the similarity between the values for the 100% glycerol solution absorption when comparing the regular Celite® and the sol–gel supported Celite® (Fig. 8), indicating that the difference caused by the sol–gel is most prominent when water is present in the equilibrating solution. According to Clifford and Legge, both contact angle measurements and TGA indicate that PTMS–TMOS sol–gels are hydrophobic and that increasing the proportion of PTMS increases the hydrophobicity of the sol–gels [20]. Therefore, the hydrophobicity of the sol–gel appears to have a stronger effect when pure water is used as the equilibrating solution rather than when glycerol–water solutions are used.

These phenomena are important in a biodiesel production process since glycerol is a by-product of the reaction. The highly absorptive properties of the Celite® sol–gel will inhibit the lipase and prevent the reaction from proceeding. Care must be taken to ensure that glycerol is removed when the reaction is run on a continuous basis. However, at low levels of glycerol (i.e. 10% by volume), very little glycerol is absorbed in the Celite® sol–gel material (approximately 2.5% by mass), so this phenomena becomes more of a concern as the concentration of glycerol increases.

4. Conclusions

A procedure to immobilize lipase in sol–gels supported on Celite® R632 was developed and shown to be valuable for the transesterification of triolein to produce methyl oleate. Three main properties were considered for comparison: protein loading, enzymatic activity, and glycerol–water adsorption, for four different sol–gel preparations: unsupported sol–gel low protein level (U-SG-4), Celite® sol–gel low protein level (C-SG-4), unsupported sol–gel high protein level (U-SG-12), and Celite® sol–gel high protein level (C-SG-12).

Based on the amount of protein retained with respect to that loaded onto the sol–gel, the immobilization procedure is more effective (i.e. incomplete protein immobilization is minimized) at the low protein levels. The water absorbed by the Celite® during sol–gel preparation must be removed as it inhibits the lipase–the maximum production of methyl oleate (80% in 6 h) was achieved

with the high protein content Celite® sol–gel (C-SG-12) after a pre-drying step. Over a 1.5-year time frame, both the unsupported and Celite® supported sol–gels exhibited high stability when stored at 4 °C. The sol–gel was found to provide a protective barrier that inhibits the absorption of the glycerol–water solution. Therefore, the removal of glycerol is key in preventing the inhibition of the lipase, but is only a concern at very high levels of glycerol (approximately 75% glycerol by volume).

Based on this study, a Celite® supported sol–gel immobilized lipase was developed that achieves high production of methyl oleate in a short reaction time. Although the immobilization procedure is straight-forward, some subtleties of the new material exist including a desirable pre-drying step to remove water absorbed during immobilization, and the potential for glycerol–water absorption when high quantities of glycerol are present. In addition to the high conversion, the immobilization regime is highly stable over 1.5 years without sophisticated storage requirements.

Acknowledgements

This work was supported by the Natural Sciences and Engineering Research Council (NSERC) in the form of a Postgraduate Scholarship to SMM and a Discovery Grant to RLL. We thank Novozymes North America for supplying samples of the lipase formulation and World Minerals for supplying samples of the Celite® support materials.

References

[1] L. Sarda, P. Desnuelle, Action de la lipase pancréatique sur les esters en émulsion, Biochim. Biophys. Acta 30 (1958) 513–521.
[2] K.R. Jegannathan, C. Eng-Seng, P. Ravindra, Renew. Sustain. Energy Rev. 15 (2011) 745–751.
[3] M.T. Reetz, A. Zonta, J. Simpelkamp, Biotechnol. Bioeng. 49 (1996) 527–534.
[4] M.T. Reetz, Adv. Mater. 9 (1997) 943–954.
[5] D. Pirozzi, E. Fanelli, A. Aronne, P. Pernice, A. Mingione, J. Mol. Catal. B: Enzym. 59 (2009) 116–120.
[6] D. Koszelewski, N. Muller, J.H. Schrittwieser, K. Faber, W. Kroutil, J. Mol. Catal. B: Enzym. 63 (2010) 39–44.
[7] K. Kawakami, Biotechnol. Tech. 10 (1996) 491–494.
[8] K. Kawakami, S. Yoshida, J. Ferment. Bioeng. 82 (1996) 239–245.
[9] A. Robles-Medina, P.A. González-Moreno, L. Esteban-Cerdán, E. Molina-Grima, Biotechnol. Adv. 27 (2009) 398–408.
[10] L. Fjerbaek, K.V. Christensen, B. Norddahl, Biotechnol. Bioeng. 102 (2009) 1298–1315.
[11] D. Ganesan, A. Rajendran, V. Thangavelu, Rev. Environ. Sci. Biotechnol. 8 (2009) 367–394.
[12] Y. Shimada, Y. Watanabe, T. Samukawa, A. Sugihara, H. Noda, H. Fukuda, Y. Tominaga, JAOCS 76 (1999) 789–793.
[13] Y. Watanabe, Y. Shimada, A. Sugihara, H. Noda, H. Fukuda, Y. Tominaga, JAOCS 77 (2000) 355–360.
[14] Y. Watanabe, Y. Shimada, A. Sugihara, Y. Tominaga, JAOCS 78 (2001) 703–707.
[15] Y. Watanabe, Y. Shimada, A. Sugihara, Y. Tominaga, J. Mol. Catal. B: Enzym. 17 (2002) 151–155.
[16] Y. Xu, W. Du, J. Zeng, D. Liu, Biocatal. Biotransform. 22 (2004) 45–48.
[17] T. Samukawa, M. Kaieda, T. Matsumoto, K. Ban, A. Kondo, Y. Noda, H. Fukuda, J. Biosci. Bioeng. 90 (2000) 180–183.
[18] J.C. Yori, S.A. D'Ippolito, C.L. Pieck, Energy Fuels 21 (2007) 347–353.
[19] V.A. Mazzieri, C.R. Vera, J.C. Yori, Energy Fuels 22 (2008) 4281–4284.
[20] J.S. Clifford, R.L. Legge, Biotechnol. Bioeng. 92 (2005) 231–237.