Thermochimica Acta 501 (2010) 84–90

Contents lists available at ScienceDirect

Thermochimica Acta

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

Novel investigation of enzymatic biodiesel reaction by isothermal calorimetry

Lene Fjerbaek Sotoft[a,*], Peter Westh[b], Knud V. Christensen[a], Birgir Norddahl[a]

[a] Institute of Chemical Engineering, Biotechnology and Environmental Technology, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark
[b] Department of Life Science and Chemistry, Roskilde University, PO Box 260, DK-4000 Roskilde, Denmark

ARTICLE INFO

Article history:
Received 24 August 2009
Received in revised form 11 January 2010
Accepted 12 January 2010
Available online 1 February 2010

Keywords:
ITC
Biodiesel
Enzyme
Lipase
Transesterification
Reaction enthalpy

ABSTRACT

Isothermal calorimetry (ITC) was used to investigate solvent-free enzymatic biodiesel production. The transesterification of rapeseed oil with methanol and ethanol was catalyzed by immobilized lipase Novozym 435 at 40 °C. The aim of the study was to determine reaction enthalpy for the enzymatic transesterification and to elucidate the mass transfer and energetic processes taking place. Based on the measured enthalpy and composition change in the system, the heat of reaction at 40 °C for the two systems was determined as -9.8 ± 0.9 kJ/mole biodiesel formed from rapeseed oil and methanol, and -9.3 ± 0.7 kJ/mole when rapeseed oil and ethanol was used. Simple Michaelis–Menten kinetics was not an appropriate choice for describing the kinetics of this heterogeneous system. The experiments demonstrated the possibility of investigating complex reaction mixtures using ITC. Although it is possible to determine thermodynamic properties such as reaction enthalpy and reaction rate, the difficulty in actually measuring the true non-mass-transfer-limited reaction kinetics is exposed by the high time resolution of ITC.

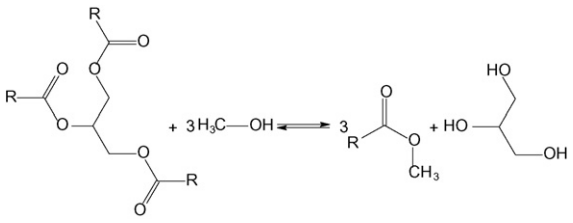
1. Introduction

Biodiesel produced from triglycerides (i.e., vegetable oils or waste fats) has been on the market for nearly 30 years as a renewable, green fuel for transportation [1]. During the last decade, the soaring price of both crude oil and of the raw materials for biodiesel have prompted research into more economically viable ways of producing biodiesel, both in order to reduce CO_2 emissions and to reduce dependency on fossil fuel. Currently, traditional homogeneous catalysts such as NaOH, KOH and H_2SO_4 are used industrially, while much new research involves heterogeneous catalysts such as immobilized bases, acids, metals and enzymes. Enzymes are commercially interesting due to a lower reaction temperature and a simpler, less energy-consuming, downstream processing.

Enzymatic biodiesel production by lipases has been thoroughly investigated in order to elucidate reaction mechanisms, reaction rates and the industrial production potential for replacement of the homogeneous chemical catalysts for the transesterification of oils/fats with methanol [2–5].

Lipases can catalyze reactions such as hydrolysis, ester synthesis, alcoholysis, acidolysis and transesterification [6]. When using an ester as substrate, the possible lipase reactions are as shown in Fig. 1. The formation of biodiesel from a triglyceride corresponds to the reaction between the ester and an alcohol, while hydrolysis can take place if water is present (see Eq. (1)).

The total reaction for production of biodiesel from a triglyceride and alcohol is:



(1)

The reaction is expected to follow a Ping Pong Bi Bi mechanism for each individual ester bond [7,8] but, for simplicity, the enzymatic alcoholysis has been fitted to steady-state Michaelis–Menten kinetic models [8,9]. The Bi Bi model describes a generalized two-substrate two-product (Bi Bi) reactions model where product formation occurs only after the formation of an enzyme–two-substrates complex. The Ping Pong Bi Bi model describes a specialized Bi Bi mechanism in which the binding of substrates and release of products is ordered. It is a Ping Pong mechanism because the enzyme shuttles between a free and a substrate-modified intermediate state. In other words, one substrate binds to the active site, is transformed into a product and that product is released, before the second substrate binds to the site, is transformed into the second product and that product is then finally released [10].

Both models, however, are based on the assumption of a single homogeneous phase in which the enzymes are dissolved, and therefore, mass-transfer limitations are avoided. The most promis-

* Corresponding author. Tel.: +45 6550 7443; fax: +45 6550 7354.
E-mail address: lfj@kbm.sdu.dk (L.F. Sotoft).

0040-6031/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.tca.2010.01.014

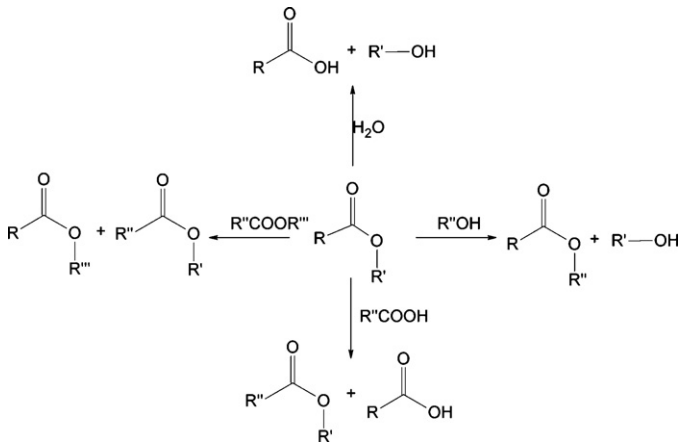


Fig. 1. Reaction scheme for lipases acting on esters.

ing use of enzymes for industrial biodiesel applications is with solvent-free conditions and with enzymes immobilized (imm.) on an inert carrier for reuse [4]. The solvent-free conditions give a two-liquid-phase system, since oil and methanol are immiscible, and the immobilized catalyst and the two immiscible substrates have a high potential for mass-transfer limitations. Thus the assumption that a homogeneous reaction phase exists and that mass-transfer limitations are not present could lead to erroneous kinetic analysis and flawed reactor design.

Currently, the kinetics of biodiesel formation are characterized and analyzed with respect to the chemical composition of the reaction mixture, i.e. product yield, by GC, MS or HPLC. The information gained from such experiments has a limited resolution with respect to time and to the reactions taking place, due to the relatively slow and laborious analytical methods and manual sampling.

Isothermal calorimetry (ITC), on the other hand, has recently been used successfully for in situ measurements of enzyme activity in homogeneous systems, and has yielded further information on reaction kinetics with much higher resolution than is possible with traditional analytical methods [11,12].

This study investigates whether ITC is capable of providing new information about the reactions taking place in a heterogeneous system when immobilized lipases are used for solvent-free biodiesel production. The systems chosen for this investigation are biodiesel produced from rapeseed oil and either methanol or ethanol catalyzed by the commercial biocatalyst Novozym 435 from Novozymes containing a *Candida antarctica B* lipase immobilized on an acrylic resin.

2. Experimental methods

2.1. Materials

The rapeseed oil was refined industrial-grade quality for biodiesel production kindly provided by Emmelev A/S, Denmark. The enzymes, Novozym 435 with 1–2 wt% water, and the inert carrier used for Novozym 435, Lewatit VP OC 1600 (60 wt% water), were kindly provided by Novozymes, Denmark. The diameter of the carrier ranges from 0.32 mm to 1.0 mm [13]. Novozym 435 consists of 0.3–0.9 mm acrylic resin beads with immobilized *C. antarctica B* lipase. Methanol (Rathburn, Denmark), isopropanol (Rathburn, Denmark), *n*-hexane (Rathburn, Denmark) and acetonitrile (Rathburn, Denmark) were HPLC-grade, while ethanol was 99.99% (Danish Distillers A/S, Denmark). Water was of ultrapure quality.

2.2. HPLC

The method used to analyze the content of triglycerides and biodiesel esters was a RP-HPLC gradient method with three eluents, as described by Holcapek et al. [14]. All solvents were filtered through a 0.45 μm filter before use. The instrument used was an Agilent 1200 series (Agilent, Denmark) with quaternary pump and UV-detection at 205 nm. Flow-rate was 1 mL/min, injection volume 10 μL, and the column was a Phenomenex Luna 3 μ C18(2) 100A at a temperature of 40 °C. Sample preparation was done by diluting a 27 μL sample into 10 mL 5:4 (vol/vol) isopropanol:*n*-hexane: 1 mL of this solution was then transferred into a vial after passing a 0.45 μm filter and placed in the HPLC autosampler for analysis. The method detects individual fatty acids, monoglycerides and diglycerides, triglycerides and fatty acid methyl esters (biodiesel).

2.3. Isothermal calorimetry

Currently, calorimetry has been used for the determination of heat of combustion and temperature properties of biodiesel [15,16], but enzymatic biodiesel production has not yet been investigated with isothermal microcalorimetry.

In isothermal calorimetry, the heat requirement (Q) needed to keep a reaction chamber at a specific temperature is measured as a function of time (t). The heat as a function of time is the power (P) put into or removed from the system to keep it isothermal (see Eq. (2)). When looking at a single chemical reaction in a batch reactor, Q is proportional to the apparent heat of reaction (ΔH_{app}). In a well-mixed batch reactor, the number of moles reaction (n) is equal to concentration ($[C]_{total}$) times mixture volume (V) (see Eq. (3)).

$$\text{Power} = \frac{dQ}{dt} \tag{2}$$

$$Q = n \cdot \Delta H_{app} = [C]_{total} \cdot V \cdot \Delta H_{app} \tag{3}$$

where: n = mole product, $[C]$ = product concentration, and V = suspension volume in the calorimetric cell.

Differentiation of Eq. (3) leads to Eq. (4), and rearrangement of Eq. (4) leads to Eq. (5) for a batch reaction. This correlates the reaction rate $\left(d[C]_{total}/dt\right)$ linearly to the power signal (dQ/dt).

$$\frac{dQ}{dt} = \frac{d[C]_{total}}{dt} \cdot V \cdot \Delta H_{app} \tag{4}$$

$$\nu = \frac{d[C]_{total}}{dt} = \frac{1}{V \cdot \Delta H_{app}} \cdot \frac{dQ}{dt} \tag{5}$$

The apparent heat of reaction (ΔH_{app}) is the sum of all effects taking place in the reaction chamber consuming or liberating heat. These can be effects such as heat of reaction, dilution, absorption, vaporization, and mixing (see Eq. (6)).

$$\Delta H_{app} = \Delta H_{reaction} + \Delta H_{dil} + \Delta H_{ab} + \Delta H_{vap} + \Delta H_{mix} \tag{6}$$

So even though the size of reaction enthalpy ($\Delta H_{reaction}$) is in most cases the dominant factor, it is still important to have knowledge of all the effects involved in the reaction in order to make the appropriate corrections; for instance by means of a baseline experiment to eliminate all the factors except the one being quantified. In this case, it is the reaction enthalpy ($\Delta H_{reaction}$).

For enzymatic reactions, kinetic constants for first-order Michaelis–Menten reactions can be determined by ITC [11], and other reaction mechanisms can also be investigated [17]. Microcalorimetry, however, will not be able to elucidate complex reaction mechanisms such as a Ping Pong Bi Bi mechanism directly. The aim, therefore, was to investigate whether the method is useful for monitoring the reaction in progress rather than for determining specific kinetic rate parameters. Furthermore the use

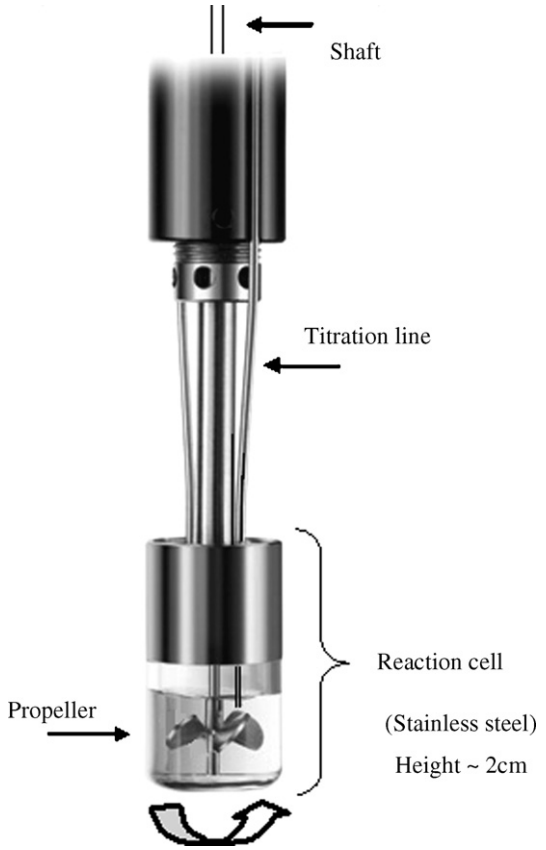


Fig. 2. Thermometric reaction cell.

of microcalorimetry is not dependent on a homogeneous phase as are traditional analytical methods.

The advantages of in situ, high-resolution ITC outweigh the drawbacks, since a great deal of information can be gained from measurement of the total activity by ITC compared with low time-resolution analysis by such methods as HPLC after sampling.

ITC was carried out in a Thermometric 2277 thermal analysis monitor (TAM, TA Instruments, USA) with four separate reaction cells and an individual reference chamber, equipped with a Thermometric 5510 pre-thermostat. The heat transfer (power signal) from each cell was logged with a PC. The samples were in 1 mL stainless-steel cells mounted on type 2250 titration units (see Fig. 2). The apparatus was equilibrated at 40 °C and calibrated up to 1000 μW by built-in electrical heaters. Measurements were carried out continuously at 15-s intervals and 5 s mean.

Reactions were carried out batchwise with stoichiometric amounts of MeOH and oil, and 10 wt% based on oil weight of the biocatalyst. The reaction mixture was composed of two immiscible liquid reactants with a solid heterogeneous catalyst. For improved mass transfer of reactants to enzymes, the experiments were carried out by continuous mixing with 113 rpm stirring using a three-bladed propeller in the reaction chamber (see Fig. 2). A syringe was used to inject methanol through the titration line during development of the ITC process.

In order to confirm that the total heat and the reaction progress were correlated, ITC experiments were carried out, where samples for HPLC analysis were withdrawn. A 50 μL sample was then taken from the ITC reaction cell with a syringe via titration line and transferred to an Eppendorf tube. Three samples were taken during an experiment and once at the end of the experiment if sampling was carried out.

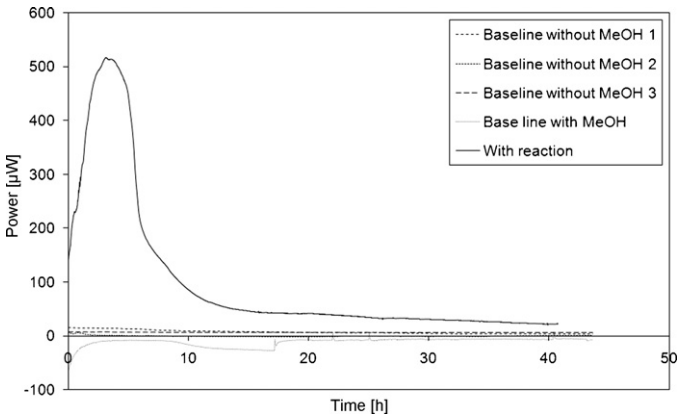


Fig. 3. Raw data from three baseline experiments without addition of methanol and one baseline experiment with addition of methanol compared with an experiment with enzymatic reaction for comparison of power signal range.

3. Results and discussion

3.1. Baseline correction

In order to compensate for all other effects than enzyme activity in an ITC experiment, a baseline correction has to be performed for the ITC trials. The baseline correction experiments were carried out with oil mixed with inactivated biocatalyst beads. Biocatalyst beads were needed in the baseline experiments due to the heat of absorption of oil, as described in Section 3.2. Three repetitions of baseline experiments can be seen in Fig. 3, where data from a biodiesel reaction with enzymatic activity are also included.

A strong signal is generally observed for experiments with enzymatic reaction. This is because no solvent is used and therefore the substrate concentrations are high, leading to a strong, easily detectable signal for the reaction system compared with enzymatic reactions taking place in diluted systems.

Inactivation was tested by measuring whether the enzymes were able to catalyze biodiesel formation for >40 h under standard reaction conditions. For inactivation, treatment at 20 °C with MeOH for 1 h followed by drying at 105 °C for 3 h was tested and found to be the most effective and efficient way to inactivate the enzymes.

MeOH inactivates the enzymes when it is the only solvent present. During reaction, where the enzymatic activity must be preserved (see Section 2.3), only stoichiometric amounts of methanol to oil are used, where the volume ratio of MeOH to oil is 1:8. Oil is therefore the dominant liquid phase and covers the enzymes on the hydrophobic carrier, so that MeOH concentration in the vicinity of the enzymes does not reach harmful inactivating levels but diffuses through the oil phase to act as a substrate in the formation of biodiesel.

The water activity in the reaction system is known to affect the lipase activity and reaction rate [18,19], and the presence of water in an enzyme suspension can influence ITC measurements. This was tested and it was found that dried inactivated Novozym 435 did not lead to significant differences compared with “wetted” inactivated Novozym 435, and can be used for baseline experiments after drying without a need to restore original water activity.

Baseline experiments with methanol (see Fig. 3), showed large power signal size variations and instability during the experiment due to what is assumed to be evaporation and condensation of methanol and bubble bursts (Fig. 3). These problems appear only when methanol is present, but not consumed. Due to the large short-term power variations, but low total influence, it was decided not to include methanol in the baseline correction, since the cor-

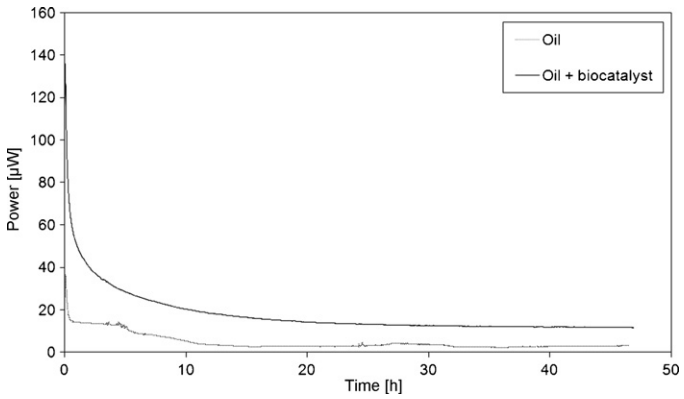


Fig. 4. Raw data from measurement of pure oil (black line) and oil plus active Novozym 435 (grey line). Time at start of stirring is 0 h.

rection with methanol in the baseline experiments actually led to a larger uncertainty than when methanol was not included.

3.2. Absorption, triglyceride hydrolysis and equilibration time

During the ITC, the thermal equilibration lasts approximately 20 min before the signal stabilizes at the baseline. The signal when having either pure oil or oil and active Novozym 435 in the chamber is shown in Fig. 4. The time from mixing all reactants in the cell until stirring begins is approx. 20 min. From this point onward, all curves shown are corrected with respect to time, where time = 0 h is when the chamber is lowered and stirring commences.

For oil alone, the energy level reached equilibrium after 0.5 h, while the mixture of oil and Novozym 435 had not reached equilibrium after 10 h. This shows that the absorption and intrusion of oil into the particles, and possibly enzymatic activity, i.e., the hydrolysis of oil with water present in the beads, is a relatively slow process.

3.3. Modeling of absorption of oil into biocatalyst

The absorption of oil into the biocatalyst was modeled using a model for diffusing substances leaving or entering a sphere [20] to further support the claim that Fig. 4 is indeed the result of diffusion kinetics. In this case, it can be seen either as oil diffusing into an air phase (entering a sphere) or as air diffusing through oil (leaving a sphere). A diffusion coefficient for air diffusing through oil is therefore estimated by use of the Stokes–Einstein equation [21]. For comparison, a modeling of methanol diffusing into oil-filled pores was also carried out.

The model is based on calculating the dimensionless mass (ω) by Eq. (7), by finding the root β of Eq. (8) with the data from Table 1. The solution is found numerically using Mathcad 14.0.

$$\omega = 1 - \sum_{i=1}^{n} \left[\frac{6(r \cdot k_c/D)^2 \cdot e^{(\beta^2 \cdot D \cdot t/r^2)}}{\beta^2(\beta^2 + (r \cdot k_c/D)^2 - (r \cdot k_c/D))} \right] \quad (7)$$

Table 1
Data for modeling.

Factor			
Particle diameter	r_p	0.40–0.45	mm
Porosity	ε	0.3–0.5	–
Tortuousity	τ	3–4	–
Temperature	T	40	°C
Density, oil	ρ	913	kg/m^3
Dynamic viscosity oil	μ	33.301	cP

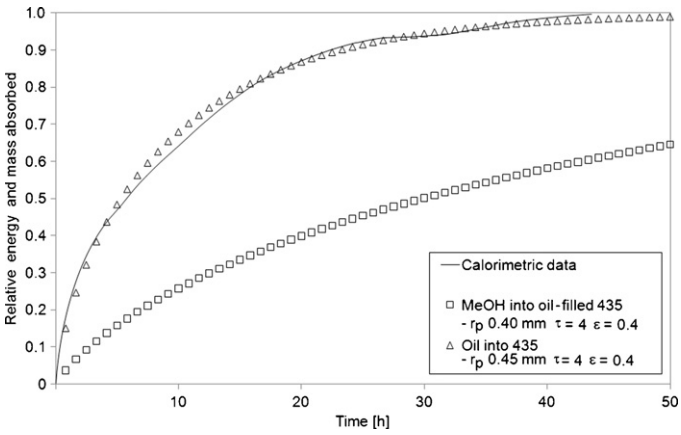


Fig. 5. Absorption into spherical biocatalyst beads as a function of time. Experimental data (solid line) are calorimetric energy. Models are represented by points (triangles and squares).

$$\beta \cdot \cot(\beta) + \frac{r \cdot k_c}{D} - 1 = 0 \quad (8)$$

where: r = particle radius, k_c = mass-transfer coefficient, D = binary diffusion coefficient, β = root of function, and t = time.

The result of modeling the absorption into the sphere is shown in Fig. 5. The calorimetric power signal and integrated heat of the absorption can be seen as an indicator of the overall absorption process.

As can be seen, the model predicts the experimental data closely for a particle radius of 0.45 mm when comparing the relative energy of absorption data from ITC with the relative mass data from the model. It can therefore be concluded that the observed heat is from the mass-transfer-limited absorption process.

3.4. ITC reaction strategy for a heterogeneous catalytic system

The general procedure for ITC is to start the reaction by injection of either the catalyst or one of the reactants to the reaction cell after equilibration of the cell with the remaining reactants and possibly a catalyst. That strategy could not be applied here.

Instead, mixing of all reactants and the catalyst prior to equilibration is the best solution for this reaction system. This can be justified since the reaction period during equilibration accounts for <1% of the total reaction time due to the relatively low reaction rate. The evidence is presented in the following section.

The diameter of the solid biocatalyst beads investigated makes post-equilibration addition impossible. Therefore, the biocatalyst must be in the reaction cell during equilibration. The normal procedure would then be to add one of the reactants by injection after equilibration. If oil is to be injected to start the reaction as a reactant, MeOH and EtOH would be present as the only liquid during equilibration and would potentially deactivate the enzymes (see Section 3.1). Oil must therefore be present in the reaction cells during equilibration.

Injection of methanol after equilibration was therefore tested following mixing of all reactants and enzymes prior to equilibration. Methanol injection in the baseline correction with inactive enzymes was also tested if that was an option. The results are shown in Figs. 6 and 7.

It is clear that the injection of methanol leads to a relatively large undesirable energy consumption (Fig. 6) compared to initial mixing, and the injection is not reproducible (Fig. 7). As seen, this affects the calorimetric signal to a much greater extent than when all reactants are mixed initially.

The experiments are run for a minimum of 40 h to obtain full conversion, due to the low reaction rate, so mixing of all reactants

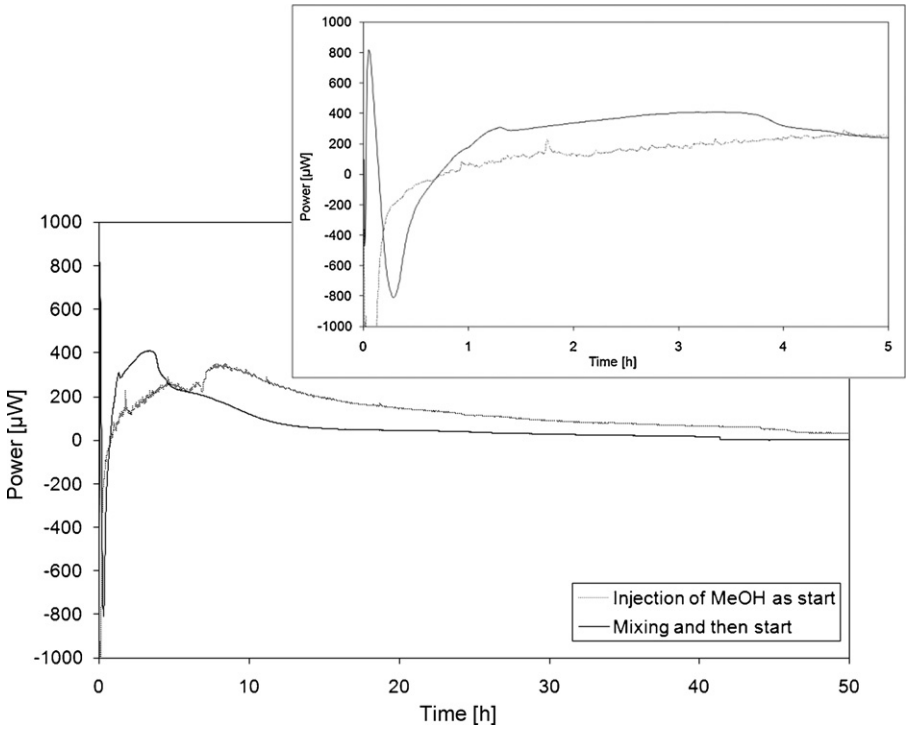


Fig. 6. Calorimetric experiment, raw data with experimental comparison of initial mix of all reactants (control) and injection of methanol to initiate the reaction. $T = 40\,^{\circ}C$. A positive value on the *y*-axis indicates an exothermic reaction. Insert shows the initial 5 h in greater detail.

prior to equilibration will give 20 min of reaction without any useful power signal. This is <1% of the total reaction time. Therefore, the best ITC reaction strategy for this system is to mix all reactants and enzymes before equilibration and accept that the initial ~20 min of the reaction cannot be monitored.

3.5. Correlation between ITC experiments and reactant conversion

Identical ITC experiments with and without sampling for HPLC analysis were carried out to confirm the hypothesis that the heat is linearly correlated to the conversion of triglycerides into biodiesel. The heat (integrated area under a power curve) must be measured in an undisturbed system without sampling, while samples must be withdrawn in an identical experiment for HPLC analysis. Afterwards, these two experimental results are combined. It can be seen from Fig. 8a that the heat of the sampling experiment is lower due to disturbance of the system and removal of reaction mixture due to the sampling, which is why an undisturbed experiment is needed. Quantification by HPLC was carried out for fatty acid esters (biodiesel) (see Fig. 8b), while the level of intermediates was below quantification level.

The integrated area under the curve varies with content, as shown in Fig. 8b, and a linear correlation is observed. Four repetitions are shown with the regression line (solid black) and confidence interval (dotted black line). The linear regression model does not start at 0% biodiesel, since some reaction has taken place after mixing of all reactants and enzymes and before the power signal can be integrated. This causes the offset from the origin (0.0), but does not influence the signal correlation.

Traditional enzymatic biodiesel experiments involving batch reactors, i.e., 20-mL flasks, were carried out for up to 50 h with shaking as the only mixing. Results from a typical shaking flask experiment are shown in Fig. 9. Samples were withdrawn manually from the flask and then analyzed by GC or HPLC. The results are generally from a sampling rate of a maximum of 20 samples. This does not give a high resolution of the change in concentration against time compared with 5-s intervals between sampling with

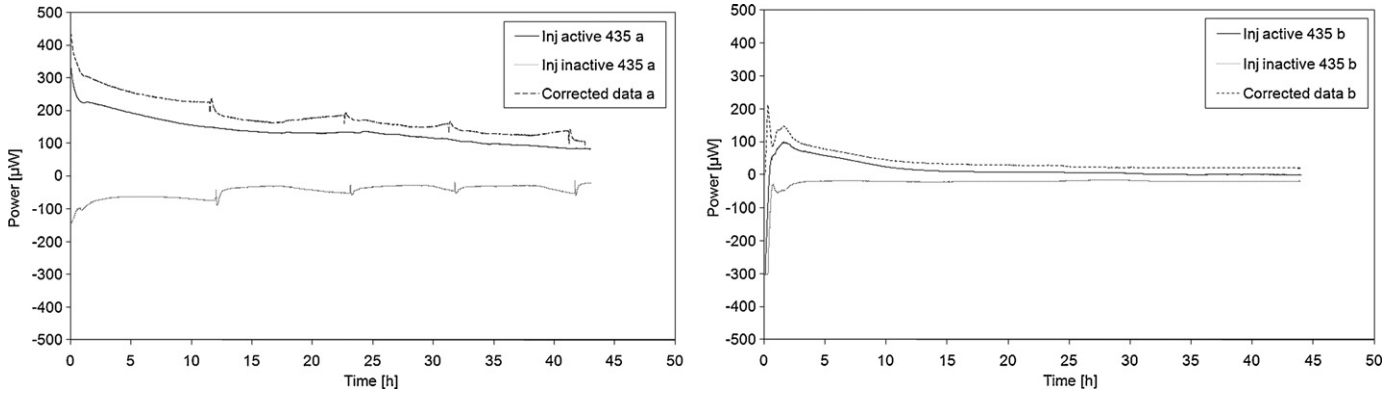


Fig. 7. Calorimetric experiment, raw data with injection of methanol into active and inactivated Novozym 435 and the calculated corrected data when using injection into inactivated Novozym 435 as a baseline experiment and subtracting data from active enzyme from the data the baseline experiment. Two repetitions (a and b) are shown.

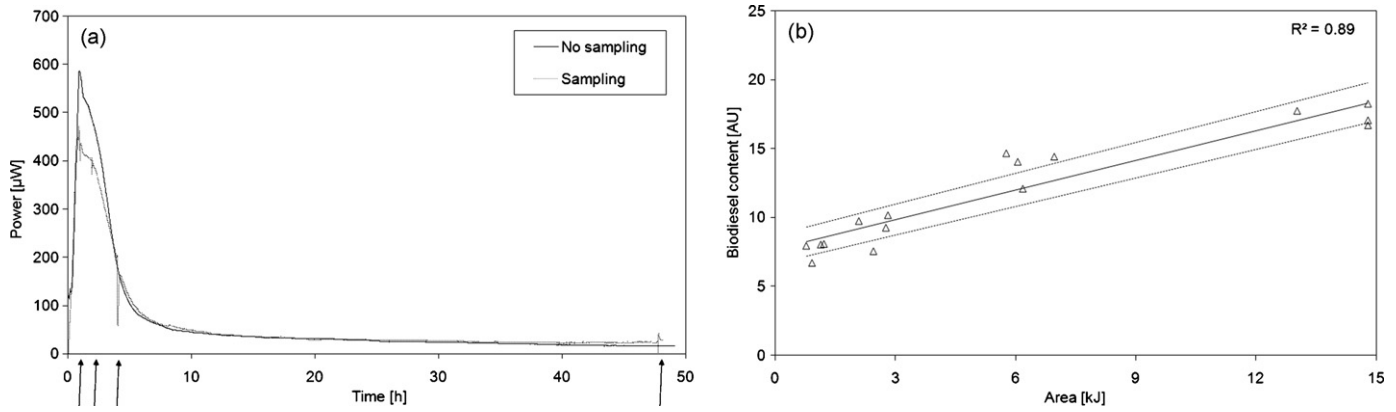


Fig. 8. (a) Comparison of TAM experiments with and without sampling. The four arrows indicate samples. (b) Content of biodiesel as measured by HPLC peak area vs. integrated area from ITC with data points (triangles), regression line (solid black line) and confidence interval (dotted black line).

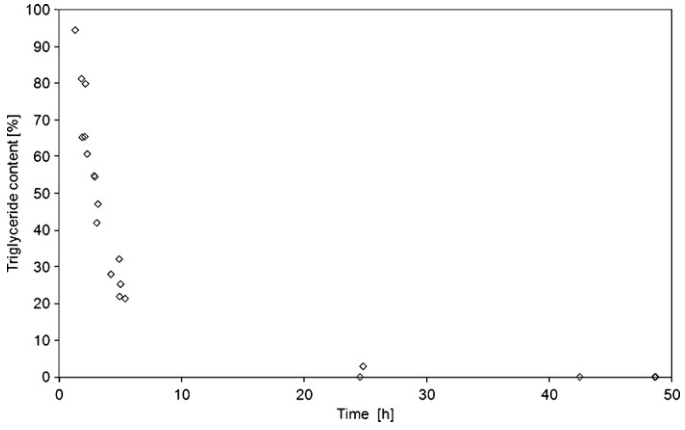


Fig. 9. Content of triglycerides (TG) as a function of time.

ITC measurements. Even so, HPLC can be used to check whether the reaction rate is proportional to the heat flow in the calorimetric vessel during ITC measurements.

Since linear behavior is observed, the ITC system can be expected to measure the heat of reaction of the conversion of triglycerides into biodiesel. Based on this, reaction enthalpy and rate can now be calculated based on HPLC results and investigated further.

3.6. Determination of the reaction enthalpy

Based on the experiments above, it can be assumed, within the limits of experimental error, that the energy released during the ITC experiment with baseline correction is the heat of reaction released by the conversion of rapeseed oil into biodiesel. A typical experiment used for reaction enthalpy is shown in Fig. 10a. The calculation of reaction enthalpy is carried out from Eq. (9) as the sum of the integrated power signal divided by the number of moles of MeOH reacted.

$$\Delta H_{\text{app}} = \frac{\sum_{i=2}\left(\frac{P_i+p_{i-1}}{2}(t_i - t_{i-1})\right)}{n_{\text{MeOH}}} \tag{9}$$

With the reaction enthalpy, the reaction rate can be calculated for each data point of measured power according to Eq. (6) from the constant suspension volume and after calculation of ΔH_{app} for the experiment (see Fig. 10a). The resolution of the reaction progression by ITC is higher and much clearer by this technique than by traditional accumulating methods such as HPLC.

Based on the ITC experiments, the reaction enthalpy at 40 °C was found to be −9.8 ± 0.9 kJ/mole biodiesel formed from the transesterification of rapeseed oil with methanol. This value compares well with what can be predicted by using group contribution correlations such as Benson's method, which yields −16.1 kJ/mole biodiesel at 40 °C [21] and −4.0 kJ/mole using an Aspen Plus estimation with database values for triolein as substrate and methyl oleate as FAME product.

By carrying out similar measurements using ethanol instead of methanol, the enthalpy of reaction at 40 °C was found to be −9.3 ± 0.7 kJ/mole, compared with the value predicted by Benson's method, which is −16.3 kJ/mole at 40 °C [21] and −3.1 kJ/mole using Aspen Plus.

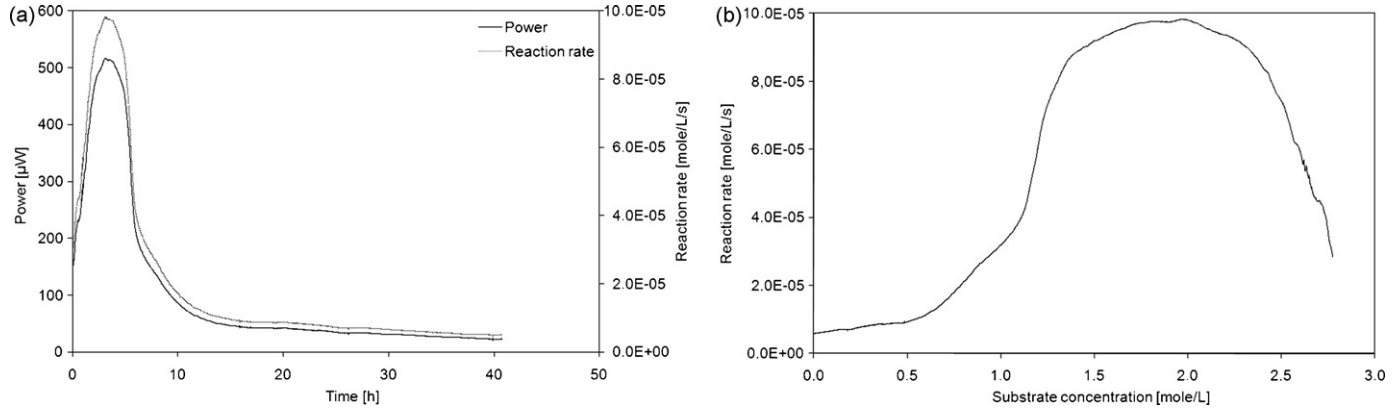


Fig. 10. (a) Raw data from the measurement of oil, methanol and 10 wt% Novozym 435 based on oil weight (left *y*-axis) and calculated reaction rate (right *y*-axis). (b) Reaction rate vs. calculated substrate concentration.

The values measured by ITC are therefore in the range of estimated values (within 50%) of methods that are widely accepted and used. ITC is thus a useful method for measurement of relatively small reaction enthalpies such as here from enzymatic reactions in complex reaction mixtures. The results show that the measured reaction enthalpies of methanol and ethanol are of comparable size despite the different volatility of the two substrates and the operational problems relating to this. This indicates that the choice of ITC reaction strategy is valid.

3.7. Mass-transfer limitation and kinetics of the reaction system

With an estimated reaction enthalpy and volume of the reaction system, the apparent reaction rate can be calculated using Eq. (5). The reaction rate is depicted as a function of calculated substrate (methanol) concentration to investigate kinetic considerations (see Fig. 10b). Substrate concentration and reaction rate are calculated based on integrated calometric data [11]. The maximum power signal is not reached until after approx. 4 h of reaction and declines rapidly after 10 h, so the reaction has a long initial phase and a very short span of maximum reaction rate. Such changes appear clearly in ITC and have not previously been reported in the literature. When comparing this with the data shown in Fig. 4 for the absorption of rapeseed oil into Novozym 435, the reason for this behavior is most likely due to the complex nature of the reaction system. Initially, the carrier pores slowly fill with reactants of which rapeseed oil by volume is clearly the largest component (volume ratio of oil to methanol is 8:1). The oil thus is most likely to fill the pores of the hydrophobic carrier. In order for a reaction to occur, the methanol has to dissolve and diffuse through the highly viscous oil toward the immobilized enzymes. This is a fairly slow process, as seen from the modeling in Fig. 5. As the reaction proceeds, the oil is turned into less viscous biodiesel and glycerol and, as methanol is highly soluble in glycerol, this facilitates the transport of methanol into the now partly glycerol-filled pores, leading to a faster reaction. At some point, the situation is reversed as glycerol and biodiesel become the most prominent components in the carrier pores, and triglycerides have to diffuse through this mixture in order for a reaction to occur.

Product inhibition on the enzymes from glycerol has previously been documented [22,23], while no biodiesel inhibition of the enzymes has been reported [4]. The glycerol product inhibition has proven not to be strict product inhibition, but is more likely to be due to mass-transfer limitations caused by glycerol blocking the carrier pores [7,24].

These effects in combination lead to the observation of a rapidly decreasing reaction rate after approx. 6 h of reaction. The total outcome of these effects is easily detected by ITC because of the method's high resolution, whereas they cannot be observed and described methodically by traditional biodiesel experiments, i.e., shaking flasks with time-consuming sampling and analysis.

The overall conclusion of the calorimetric experiments is that it is therefore not possible to actually measure the strict reaction kinetics for the conversion of rapeseed oil into biodiesel using Novozym 435 in a non-solvent system directly, as the reaction will be severely mass-transfer-limited, as previously claimed by Fjerbaek et al. [4]. Product inhibition by glycerol is also a possibility.

ITC clearly shows that solvent-free biodiesel reaction rate by immobilized Novozym 435 is not a hyperbolic function of substrate concentration (see Fig. 10b), as would be expected for systems following Michaelis–Menten kinetics. Therefore, the outcome of traditional kinetic studies, i.e., Michaelis–Menten kinetics based on advanced statistically designed experiments, will not give a true theoretical description of the system, but merely provides suitable mathematical models with a good fit to the experimental data.

Therefore, further calorimetric work is required for clarification and the generation of more knowledge about these processes on an experimental as well as a model basis.

4. Conclusions

The ITC experiments carried out clearly demonstrate the possibilities and limitations of investigating complex reaction mixtures using ITC. Careful baseline correction and initial testing make it possible to determine the thermodynamic properties such as the reaction enthalpy, and the difficulty in actually measuring the true reaction kinetics is exposed by the high time resolution of ITC.

ITC clearly shows that solvent-free biodiesel reaction rate with imm. Novozym 435 is not a hyperbolic function of substrate concentration, as would be expected for systems following Michaelis–Menten kinetics. Thus, at this point, the main results must be that determining the true reaction kinetics for the solvent-free reaction between rapeseed oil and methanol or ethanol using Novozym 435 is probably not possible, but that the heat of reaction at 40 °C for the two systems can be determined with reasonable accuracy to be -9.8 ± 0.9 kJ/mole biodiesel formed from rapeseed oil and methanol, and -9.3 ± 0.7 kJ/mole when rapeseed oil and ethanol is used.

Acknowledgements

This work is supported by The Danish Council for Strategic Research and The Carlsberg Foundation.

References

[1] M. Mittelbach, C. Remschmidt, Biodiesel: The Comprehensive Handbook, 3rd ed., Martin Mittelbach Publishing, Graz, Austria, 2006.
[2] C.F. Torres, A.M. Tore, T. Fornari, F.J. Senorans, G. Reglero, Biochem. Eng. J. 34 (2007) 165–171.
[3] Y. Watanabe, Y. Shimada, A. Sugihara, H. Noda, H. Fukuda, Y. Tominaga, J. Am. Oil Chem. Soc. 77 (2000) 355–360.
[4] L. Fjerbaek, K.V. Christensen, B. Norddahl, Biotechnol. Bioeng. 102 (2009) 1298–1315.
[5] Y. Shimada, Y. Watanabe, A. Sugihara, Y. Tominaga, J. Mol. Catal. B: Enzym. 17 (2002) 133–142.
[6] V.M. Balcao, A.L. Paiva, F.X. Malcata, Enzyme Microb. Technol. 18 (1996) 392–416.
[7] V. Dossat, D. Combes, A. Marty, Enzyme Microb. Technol. 30 (2002) 90–94.
[8] A.L. Paiva, V.M. Balcao, F.X. Malcata, Enzyme Microb. Technol. 27 (2000) 187–204.
[9] S. Hari Krishna, N.G. Karanth, Biochim. Biophys. Acta 1547 (2001) 262–267.
[10] M. Biselli, U. Kragl, C. Wandrey, in: K. Drauz, H. Waldmann (Eds.), Enzyme Catalysis in Organic Synthesis, 2nd ed., Wiley-VCH, Weinheim, Germany, 2002, pp. 185–257.
[11] M.J. Todd, J. Gomez, Anal. Biochem. 296 (2001) 179–187.
[12] F.P. Schwarz, T. Reinisch, H.J. Hinz, A. Surolia, Pure Appl. Chem. 80 (2008) 2025–2040.
[13] Lanxess, Product Information Lewatit VP 1600, http://lanxess.com/brands-products/product-search/detail/lewatitR-trial-product-1600/?tx_editfiltersystem_pi1[pointer]=32 Lanxess Deutschland (2008).
[14] M. Holcapek, P. Jandera, J. Fischer, B. Prokes, J. Chromatogr. A 858 (1999) 13–31.
[15] S.M. Akers, J.L. Conkle, S.N. Thomas, K.B. Rider, J. Chem. Ed. 83 (2006) 260–262.
[16] R.O. Dunn, J. Am. Oil Chem. Soc. 76 (1999) 109–115.
[17] R.J. Wilson, A.E. Beezer, J.C. Mitchell, W. Loh, J. Phys. Chem. 99 (1995) 7108–7113.
[18] L. Deng, X.B. Xu, G.G. Haraldsson, T.W. Tan, F. Wang, J. Am. Oil Chem. Soc. 82 (2005) 341–347.
[19] Y. Watanabe, P. Pinsirodom, T. Nagao, T. Kobayashi, Y. Nishida, Y. Takagi, Y. Shimada, J. Am. Oil Chem. Soc. 82 (2005) 825–831.
[20] J. Crank, The Mathematics of Diffusion, 2nd ed., Oxford University Press, New York, 1975.
[21] B.E. Poling, J.M. Prausnitz, J.P. O'Connell, Properties of Gases and Liquids, 5th ed., McGraw-Hill, 2001.
[22] K. Belafibako, F. Kovács, L. Gubicza, J. Hancsók, Biocatal. Biotransform. 20 (2002) 437–439.
[23] V. Dossat, D. Combes, A. Marty, Enzyme Microb. Technol. 25 (1999) 194–200.
[24] D.E. Stevenson, R.A. Stanley, R.H. Furneaux, Enzyme Microb. Technol. 16 (1994) 478–484.