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LETTER TO THE EDITOR

Online Pre-purification for the Continuous Enzymatic Interesterification of Bulk Fats Containing Omega-3 Oil

Nuzul A. Ibrahim · Sanna i T. Nielsen · Vinotha Wigneswaran · Hong Zhang · Xuebing Xu

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Dear Sir

There has been dramatic progress in the last few years in the technology development and scientific advancement in the lipase-catalyzed modification of bulk fats such as those used for margarine production [1–5]. It has been one of the major breakthroughs that enzyme technology can be used for processing of common foods economically, and imparts other potential advantages such as less pollution, simplified processes, improved product quality, etc. The technology has been increasingly utilized in industry [6].

One of the poorly understood issues of the technology is the reduction of catalyst activity following the reuse of enzymes in industrial operations. This is different from chemical interesterification using a chemical catalyst, where no such phenomenon occurs in industrial operations. We now know that enzyme function can be affected not only by the inherent characteristics of the lipase such as thermostability and optimal water activity but also by many external factors such as material/oil quality and process parameters.

Oil quality in reality can vary with the source, and is a function of processing operations. We know a few factors are inherent to oil quality such as metal content, peroxide content, polymer content, gum content, etc. In the native state, enzymes are folded into a three dimensional, compact, globular and/or rod-like conformation of minimal free energy. Many of the above-mentioned agents can affect the native state and cause denaturation. Ohta et al. [7] reported that polymerization of lipase occurred due to the presence of hydroperoxides which led to lipase deactivation. Peroxide values greater than 5 mequiv/kg were found to be detrimental to enzyme activity retention [8], while, in another study, secondary oxidation products had a stronger effect than primary oxidation products on enzyme stability [9]. Furthermore, hydroperoxide compounds as well as other impurities such as polymers, gums, etc. were reported to be fully or partially absorbed by the enzyme bed [10]. All these studies suggest that better quality in terms of low oxidation and other detrimental components is needed for improved stability of the lipases during reuse. For all of these reasons, a refined, bleached, and deodorized (RBD) oil has been recommended for practical operations of the enzymatic interesterification processes [11].

To improve nutritional quality, the need to increase the omega-3 polyunsaturated fatty acyl content of bulk oil for products such as margarine has gained attention. This is particularly relevant to the use of enzyme processing for such modifications where milder conditions of the enzyme process can be really beneficial for the protection of omega-3 oils against oxidation. Omega-3 oils are very sensitive to oxidation and can occur during the process operation. Therefore, even using an RBD oil may be insufficient to maintain the enzyme stability, as demonstrated in a recent study where the increase of omega-3 oil in the substrate reduced the stability of the lipase used [12]. Therefore, an additional solution is imperative. One solution is an on-line monitoring process to closely follow the change of reaction performance [13, 14]. In addition, an on-line pre-purification step to remove impurities from the substrates prior to their introduction to the bioreactor would be useful to increase the stability of lipase since oxidation can occur in purified oils during storage.

Therefore, in this study, an online pre-purification step was implemented through a pre-column upstream of the

N. A. Ibrahim · S. T. Nielsen · V. Wigneswaran · H. Zhang · X. Xu (✉)
BioCentrum-DTU, Technical University of Denmark, Building 227, 2800 Kgs. Lyngby, Denmark
e-mail: xx@biocentrum.dtu.dk

packed bed reactor for the enzymatic interesterification. The oil blends employed as substrate contained sunflower and fish oils in order to accelerate the possible oxidation and make the performance of pre-purification significant. Two common absorbents, molecular sieves and activated carbon, were used for this purpose. In addition a bed containing spent immobilized lipase for pre-cleaning was also employed for practical process considerations. The deactivation rates of the enzyme bed with and without the pre-column were compared.

Refined, bleached and deodorized (RBD) sunflower oil was purchased from a local supermarket (peroxide value 2.6 mequiv/kg, water content 0.06 wt%, free fatty acid content 0.03%, and fatty acyl composition C16:0 6.0, C16:1 0.3, C18:0 4.6, C18:1n-9 17.8, C18:2n-6 69.2, others 2.1). RBD fish oil was obtained from Maritex AS, Sortland, Norway (peroxide value 4.1 mequiv/kg, water content 0.06 wt%, free fatty acid content 0.12%, and fatty acid composition C14:0 8.0, C15:0 0.6, C16:0 14.0, C16:1 4.6, C17:0 0.7, C17:1 0.7, C18:0 2.0, C18:1n-9 13.0, C18:1n-7 1.9, C18:2n-6 2.7, C18:3n-3 2.3, C18:4n-3 8.7, C20:1 4.2, C20:5n-3 10.4, C22:1n-11 7.6, C22:5n-3 1.3, C22:6n-3 16.2, others 1.1). The two oils were blended to give a 7/3 (v/v) composition ratio. Lipozyme TL IM, a silica granulated *Thermomyces lanuginosa* lipase, was donated by Novozymes A/S, Bagsvaerd, Denmark (water content 5.8 wt%). Adsorbents for the pre-column were molecular sieves (5 Å diameter), activated carbon (50 mesh and 900–1,100 m^2/g surface area, Sigma, St Louis, MO), and deactivated Lipozyme TL IM. The deactivation was conducted at 120 °C for 24 h. The deactivated lipase was checked through interesterification with the oil blend and no significant change of triacylglycerol profile was observed. Triacylglycerol standard was purchased from Nu-Chek Prep Inc., Minnesota, USA. All chemicals and reagents for analysis were of analytical or chromatographic grades.

The oil blend was subjected to enzymatic interesterification in a continuous packed bed reactor, a jacketed stainless steel column filled with 5.5 g Lipozyme TL IM (*Thermomyces lanuginosa*) lipase and heated to 70 °C by a circulatory water bath. The column dimension was 200 mm long and 15 mm in internal diameter and both ends were plugged with defatted cotton. An empty column of the same dimensions was connected before the enzyme bed. Both columns were heated to 70 °C by the same water bath. The columns were first flushed with five bed volumes of the oil blend for enzyme conditioning by a metering pump (Fluid Metering Inc., New York, NY) with an upward flow of approximately 1 mL/min [12]. The residence time for the enzyme bed was then adjusted to 50 min through the change of substrate feeding rate. The first sample was collected after operating for 1 h to indicate the initial activity of the enzyme, to insure that the enzyme bed was properly conditioned initially [12]. Additional samples were collected daily from the outlet of the packed bed reactor during the operation of the bioreactor system. The samples were stored at −40 °C prior to analysis. When pre-purification was implemented before the packed bed reactor, the empty column was filled with absorbents. Approximately 6 g absorbents were packed into the column in the similar way to enzyme packing. The columns were conditioned in the same way as without absorbents by quickly pumping five bed volumes of the oil blends. Both the enzyme bed and the pre-column were simultaneously conditioned during the flushing of the oil blend. Samples were collected from the packed bed outlet in the same way as described above.

The triacylglycerol profile of samples was determined using a Hitachi–Merck HPLC Series 7000 (Hitachi–Merck, Japan) system with a Supelcosil LC-18, 250 mm × 4.6 mm column and PL-ELS 2100 evaporative light scattering detector (Polymer Laboratories, Shropshire, UK). The mobile phase consisted of acetone and acetonitrile at 1.5 mL/min, with the ratio of the former to the latter varying linearly with the gradient rate of 0.5 volume change per min from 50/50 to 70/30 v/v during a 40 min analysis time. The degree of interesterification (ID) is defined as the peak ratio between two most significantly changed peaks as described elsewhere [4]. The initial ID (ID_0) is then defined as the ID value for the sample collected at 1 h. The residual activity (RA) is therefore defined as:

$$\mathrm{RA}(\%) = \frac{\mathrm{ID}_t - \mathrm{ID}_\mathrm{B}}{\mathrm{ID}_0 - \mathrm{ID}_\mathrm{B}} \times 100\% \quad (1)$$

where ID_t and ID_B are the ID at operational time t (h) and for the oil blends prior to their contact with lipase, respectively.

Both oils used in the blend were RBD with reasonably low peroxide values (2.6–4.1 mequiv/kg). From a commercial point of view, the oils would be regarded as high quality. However, sunflower oil contains about 70% linoleic acid and fish oil about 30% omega-3 polyunsaturated fatty acids. The latter is highly sensitive to oxidation. As expected, the residual activity decreased steadily during 200 h of operational time (Fig. 1) and reached 50% at the end of the operation. The phenomenon was confirmed by repeated trials. When selected absorbents were employed in the pre-column, the stability was greatly improved (Fig. 1). There were slight differences between the absorbents used. However, the improvements were generally significant.

The absorbents selected were not arrived at by extensive screening but with the simple aim to absorb polar compounds resulting from oxidation, residual metal elements,

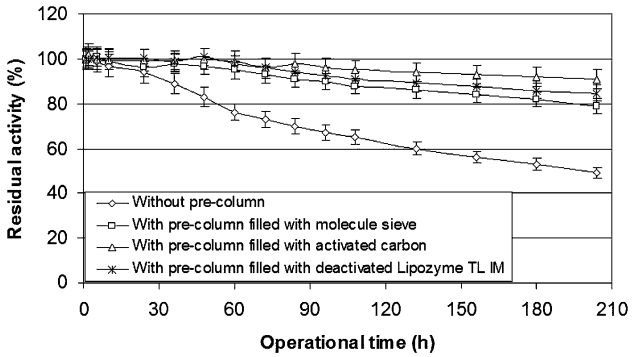

Fig. 1 Residual activities following the continuous operation of Lipozyme TL IM-catalyzed interesterification in a packed bed reactor with or without a pre-column. Reaction conditions: oil blend sunflower oil/fish oil (7/3 v/v), column temperature 70 °C, residence time 50 min, and no additional water for the oil blend. Other conditions are given in the text

gums, etc. after RBD processes. From a practical and economical point of view, the use of a spent enzyme bed as a pre-purification stage will be feasible because it serves as a waste product. This will be viable for practical processes as well. In this case, we conducted the experiments using Lipozyme TL IM artificially deactivated by heating. The absorption behaviour of the spent lipase could differ from that of the active enzyme due to differences in water content or protein structural state. After conditioning with the oil blend, we believe that the absorption performance should not be significantly different. Certainly this should be investigated further.

In the present study, the possible effect of water content on the stability was also discussed. Generally, water content has little effect on the enzyme activity for Lipozyme TL IM as demonstrated previously [2]. To reduce the effect of the water content, the conditioning by flushing with the oil blend was extended to five bed volumes from the three normally used. Both molecular sieves and activated carbon were not pre-treated by drying and used as they were. We measured the free fatty acid content for the four experimental runs for the samples after operating for 1 h, with the method previously described in [4]. There were no significant differences (1.4–1.6%), indicating a similar water activity in the enzyme bed system in the presence versus absence of the pre-columns. This also implies that there was no significant drying of the pre-column's absorbents.

Deactivation can be generally described by a first-order kinetic equation:

$$\mathrm{RA} = \mathrm{RA}_0 \times \mathrm{e}^{-k_\mathrm{d}t} \tag{2}$$

where RA_0 is initial residual activity (100% here), RA residual activity at time t, k_d the deactivation rate (h^{-1}), and t is running time (h). Upon rearrangement, this equation can be described as:

$$\ln(\mathrm{RA}) = -k_\mathrm{d}t + \ln(\mathrm{RA}_0) \tag{3}$$

Furthermore, the operational time to reach 50% residual activity ($t_{1/2}$) can be calculated as:

$$t_{1/2} = \frac{\ln(2)}{k_\mathrm{d}} \tag{4}$$

With the data in Fig. 1, linear plots can be made that correspond to Eq. 3 that yield k_d (and hence $t_{1/2}$ via Eq. 4) and $\ln(\mathrm{RA}_0)$ from the slope and intercept, respectively. Table 1 summarizes the calculation results. As seen from the table, the fitting was relatively satisfactory with R^2 values higher than 0.937. The theoretical $\ln(\mathrm{RA}_0)$ value, $\ln(100) = 4.605$, corresponds well with the calculated values. When pre-column was used, the stability was improved by 3.1, 7.4, and 4.1-fold using molecular sieves, activated carbon, and deactivated Lipozyme TL IM, respectively, in terms of k_d or $t_{1/2}$.

The capacity of the pre-column filled with the deactivated Lipozyme TL IM was also tested. The oil blend was pumped through the pre-column without passing through the enzyme beds. The pre-columns after contacting 300, 600, 900, and 1,200 kg oil blend per kg absorbent were subjected to the same experimental evaluation of stability with the same procedure described above but for 1 week. k_d was found to increase proportionally to the amount of oil fed to the column per unit mass of absorbent (Fig. 2), indicating a deterioration of the pre-column performance. However, a major decrease was found employing 800 kg/kg absorbent. This may indicate the full saturation of the pre-column's absorption sites.

Table 1 Calculation results based on the first order deactivation kinetics

	k_d (h^{-1})[a]	$\mathrm{Ln}(\mathrm{RA}_0)$[a]	R^2 [a]	$t_{1/2}$ (h)[b]
Without pre-purification	0.0037 ± 0.0002	4.595	0.984	187 ± 11
With pre-column filled with molecular sieves	0.0012 ± 0.0001	4.613	0.984	578 ± 48
With pre-column filled with activated carbon	0.0005 ± 0.00003	4.614	0.937	1386 ± 83
With pre-column filled with deactivated Lipozyme TL IM	0.0009 ± 0.00004	4.622	0.951	770 ± 34

[a] Calculation based on Eq. 3. R^2 is the linear regression coefficient representing goodness of linearities for Eq. 3 with experimental results

[b] Calculated based on Eq. 4

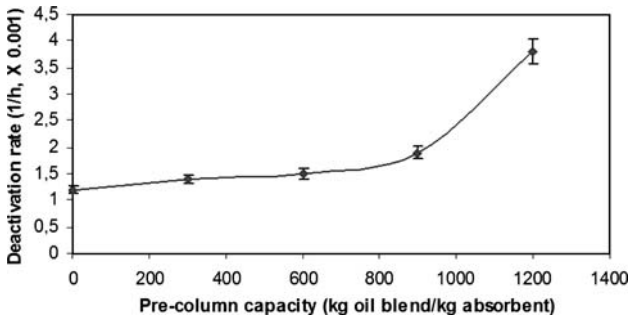

Fig. 2 Deactivation rates (k_d) versus amount of oil blend fed to pre-column filled with deactivated Lipozyme TL IM (pre-column capacity). Operational conditions given in Fig. 1

In general, this study has demonstrated the practicality of employing an online pre-purification stage for the stabilization of the enzyme bed. In particular, the use of a spent enzyme bed could be economical and practically feasible. The study used a highly unsaturated oil blend in order to make the study significant. For less unsaturated oil blends, the performance needs to be demonstrated further. The use of actual rather than simulated spent enzyme for this purpose needs to be investigated.

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