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Preparation of multipurpose cross-linked enzyme aggregates and their application to production of alkyl ferulates

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Abstract

Commercial multicomponent enzyme preparations, Ultraflo L, Depol 740L and Depol 670L, with feruloyl esterase activity, were tested for the transesterification of methyl ferulate to 1-butyl ferulate in their free and immobilized form using as a reaction system a ternary water–organic mixture consisting of *n*-hexane, 1-butanol and water. A number of factors affecting enzymes precipitation and cross-linking into cross-linked enzyme aggregates (CLEAs) have been investigated. Consecutive optimization of the precipitant type and cross-linker concentration resulted in CLEAs showing higher operational stability and synthetic activity compared to the free enzymes' forms. Under certain optimization conditions, conversion yields of 97%, 87% and 5%, were obtained by CLEAs prepared from Ultraflo L, Depol 740L and Depol 670L, respectively. The activities initially present in the three commercial preparations were completely retained after cross-linking resulting in multipurpose biocatalysts which have the potential to carry out different and independent reactions. This work is consistent to the novel CLEA concept called combi-CLEA.

Keywords: Alkyl ferulates; Feruloyl esterases; Enzyme immobilization; CLEA; Transesterification

1. Introduction

Various hydroxycinnamic acids (ferulic, *p*-coumaric, caffeic, sinapic) have widespread industrial potential by virtue of their antioxidant properties. Generally, such natural antioxidants are partially soluble in aqueous media, limiting their usefulness in oil-based processes and that has been reported to be a serious disadvantage if an aqueous phase is also present. Ferulic acid which is well known for its powerful antioxidant, photoprotective and antitumor properties [1] might find use in food, cosmetic, pharmaceutical and material industry. The modification of ferulic acid via esterification with aliphatic alcohols results in the formation of more lipophilic derivatives.

Various studies have examined the enzymatic direct esterification of ferulic acid or the transesterification using activated esters of ferulic acid as acyl donors, catalysed by purified feruloyl esterases (FAEs) or crude commercial preparations exhibiting FAE activity. FAEs which are also known as cinnamoyl esterases [E.C. 3.1.1.73] represent a subclass of carboxylic acid esterases that catalyses the hydrolysis of the ester bond between hydroxycinnamic acids and sugars present in plant cell walls. Novel FAEs purified from *Fusarium oxysporum*, *Sporotrichum thermophile* and *Aspergillus niger*, have been used in the synthesis of alkyl ferulates such as 1-propyl ferulate [2], 1-butyl ferulate [3], 1-pentyl ferulate [4], 1-glyceryl ferulate [5] and for the synthesis of sugar ferulates such as 5-*O*-(*trans*-feruloyl)-L-arabinofuranose [6] or *O*-[5-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1 → 5)-L-arabinofuranose [7]. Commercial enzyme preparations exhibiting FAE activity were also used for synthetic purposes. For example, enzyme preparations from *Humicola insolens*, *Thermomyces lanuginosus* and *A. niger* were used for the transesterification of various glycosides at their primary hydroxyl group [8]. Furthermore, a commercial enzyme preparation from *H. insolens* catalysed the asymmetric transesterification of secondary alcohols which are not natural substrates for FAEs [9]. The above reactions were performed in various reaction media that enhance the solubility of the starting reagents and at the same time shift the reaction equilibrium towards esterification.

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Recently, a carrier-free enzyme immobilization approach has attracted more attention due to clear advantages: highly concentrated enzyme activity in the catalyst, high stability and the low production cost due to the exclusion of an additional carrier [10–12]. Cross-linked enzyme aggregates (CLEAs) were first introduced by Cao et al. [13], an approach that consists of the covalent cross-linking of a precipitated enzyme. In general, proteins can be precipitated by agents such as organic solvents or inorganic salts without undergoing denaturation. Consequently, cross-linking of performed physical aggregates of enzymes constitute a simple method for the preparation of CLEAs. This technique had been successfully applied in the preparation of carrier-free enzyme immobilization of aminoacylase, a hydroxynitrile lyase and some lipases using pure enzyme preparations [14–18]. The CLEA methodology is applicable to essentially any enzyme including crude preparations, affording stable, recyclable catalysts with high retention of activity [19]. This technique is also applicable to the preparation of combi-CLEAs, containing two or more enzymes, for use in one-pot, multi-step syntheses [20]. Recently, a new approach of combi-CLEAs is aimed at turning CLEA into a multipurpose biocatalyst capable of catalyzing non-cascade reactions [21,22]. This approach is based upon the fact that CLEA can be made from heterogeneous populations of proteins/enzymes, such as the commercial enzyme preparations used in the present work.

As there is no commercial availability of a pure FAE preparation, the present work was undertaken to investigate the effect of certain factors on the preparation of CLEAs by various commercial multicomponent enzyme preparations (Ultraflo L, Depol 670L and Depol 740L) exhibiting FAE activity and their application for the transesterification of ferulic acid with 1-butanol. The produced CLEAs were investigated for other enzymatic activities simultaneously captured in the multi-enzyme complexes which have the potential for multi-step conversions in cascade or non-cascade catalysis.

2. Materials and methods

2.1. Materials

The methyl esters of hydroxycinnamic acids such as ferulic (MFA), *p*-coumaric (M*p*CA), caffeic (MCA) and sinapic (MSA) acid were purchased from Apin Chemicals Ltd. (Abingdon, UK). Carboxymethyl cellulose (CMC), birchwood xylan, laminarin and polygalacturonic acid were purchased from Sigma Chemical Co. (St Louis, USA) while unbranched arabinan (sugar beet) was obtained from Megazyme (Wicklow, Ireland). All other reagents were purchased from LabScan (Dublin, Ireland).

2.2. Enzymes

Three commercial enzymes containing FAE activity were used in the present study. According to information provided by the suppliers, Ultraflo L (Novozymes, Denmark) and Depol 740L (Biocatalysts Ltd., UK) are multicomponent enzyme preparations from *Humicola insolens* while Depol 670L (Biocatalysts Ltd., UK) is a fungal enzyme blend. These three commercial enzyme preparations were provided as a gift by the aforementioned companies.

2.3. Enzyme and protein assays

FAE activity was assayed using destarched wheat bran (DSWB) or methyl ferulate (MFA) as substrate, measuring the release of free ferulic acid at 50 °C as described previously [2]. MFA was used for the optimisation of the immobilization procedure, since it is a faster method. One unit of activity (1 U) is defined as the amount of enzyme (mg) or milliliter of enzyme solution releasing 1 μmole of free acid per minute under the defined conditions.

Detection of other enzymatic activities present in the commercial preparations such as endoglucanase, xylanase, arabinanase, β-glucanase and polygalactorunase were assayed on CMC, birchwood xylan, unbranched arabinan from sugar beet, laminarin and polygalacturonic acid, respectively, dissolved in 100 mM MOPS–NaOH buffer (1%, w/v), pH 6.0. All assays were carried out at 50 °C for 10 min (endoglucanase assay), 15 min (xylanase and polygalactorunase assays) and 60 min (arabinanase and β-glucanase assays). The release of reducing sugars was determined using the 3,5-dinitrosalicylic acid (DNS) method [23]. A total of 1 U of enzyme activity was defined as the amount of enzyme liberating 1 μmole of reducing sugars per minute.

FAE hydrolytic activity of Ultraflo L, Depol 740L and Depol 670L was tested against MFA, M*p*CA, MCA and MSA and the release of the acids measured by HPLC as previously described [2]. All assays were prepared and analyzed in duplicate, with <10% standard error for each set of results at 50 °C. The amount of free acid released was quantified against standard curves.

CLEAs assayed for FAE activity in an Eppendorf Thermomixer Comfort (Eppendorf, Germany) operating at 1400 rpm under the above-mentioned conditions.

2.4. Immobilization of enzymes

The three commercial crude enzyme preparations were immobilized using a two-step procedure, according to the method of Schoevaart et al. [19]. Precipitation (first step) is performed by adding 10 μL of enzyme solution to 90 μL of precipitant, followed by 15 min of stirring. Subsequently, the mixture was quenched with 900 μL buffer, giving complete resolvation of the protein. Samples were withdrawn before and after precipitation and assayed for FAE activity.

Cross-linking (second step) includes the addition of certain amount of glutaraldehyde (cross-linker) after the precipitation step. The samples were incubated at room temperature for 3 h and then 1.8 mL of buffer 100 mM MOPS–NaOH pH 6.0 was added. A sample was withdrawn from the resulting suspension, which contains CLEA as well as residual free enzyme, and assayed for FAE activity. Then, the CLEA is centrifuged off and again a sample is withdrawn from the supernatant which now contains only free enzyme. The difference in FAE activity between these two samples gives the CLEA activity. This approach is the most simple and accurate way of determining

the activity of the CLEA, without having to wash and redisperse, which will increase clotting and thereby mass-transport limitations [19].

For the preparative production of CLEAs of the three enzyme preparations, 100 μL of the enzyme solution to 900 μL of saturated $(NH_4)_2SO_4$, followed by 15 min of stirring. The appropriate amount of glutaraldehyde was then added (optimum concentration found for each enzyme preparation, see Section 3.2.2) and the mixture was stirred for 3 h at ambient temperature. 9 mL of buffer 100 mM MOPS–NaOH pH 6.0 were added, followed by centrifugation. After removing the supernatant, CLEAs were washed with 10 mL of buffer 100 mM MOPS–NaOH pH 6.0, followed by centrifugation and removal of the supernatant. CLEAs were freeze-dried, weighted for calculating the productivity and assayed for all enzymes activities.

2.5. Transesterification reaction

Transesterification of MFA with 1-butanol catalysed by the free and immobilized enzyme forms was performed in the ternary system consisted of *n*-hexane/1-butanol/water (47.2:50.8:2.0, v/v/v) as described previously [3]. In the reactions catalysed by CLEAs, the enzyme solution was replaced by 100 mM buffer MOPS–NaOH pH 6.0 in the required volume. All reactions (2 mL) were performed in an Eppendorf thermomixer operated at 1400 rpm and 37 °C.

Quantitative analysis of the product was performed by HPLC as described previously [3]. Yields for the synthesis of 1-butyl ferulate were calculated from the amount of methyl ferulate having reacted compared to its initial quantity. No methyl ester consumption was observed in the absence of enzyme preparation.

2.6. Operational stability of immobilized enzymes

The reusability of the immobilized enzymes was assessed by testing their remaining FAE activity after two cycles of reaction in surfactantless microemulsion mixtures (2 × 6 days). In each cycle, the reaction mixtures were centrifuged to remove the supernatants and CLEAs then washed several times with *n*-hexane and water followed by freeze-drying.

2.7. Structure of CLEAs

FEI Quanta 200 scanning electron microscope (SEM) was used for the structure analysis of CLEAs.

3. Results and discussion

3.1. Enzymatic activities of the commercial enzyme preparations

For the modification of ferulic acid towards 1-butyl ferulate our work was mainly focused on the feruloyl esterase activity present in the three commercial preparations tested. Therefore, the three preparations were tested for their hydrolytic activity against four methyl esters of hydroxycinnamic acids (Table 1).

Table 1
Hydrolysis of methyl esters of cinnamic acids by the three commercial enzymes

Substrate	Commercial enzyme preparation (U/mL)		
	Ultraflo L	Depol 740L	Depol 670L
MFA	13.57 (1.21)	10.48 (0.40)	3.49 (0.07)
M*p*CA	16.70 (1.02)	6.65 (0.03)	4.15 (0.37)
MCA	24.90 (1.24)	55.08 (0.78)	5.07 (0.02)
MSA	0.25 (0.01)	8.99 (0.01)	0.55 (0.01)
DSWB	0.59 (0.06)	0.56 (0.01)	0.36 (0.02)

Assays were performed in duplicate and standard error is presented (number in parenthesis). MFA: methyl ferulate; M*p*CA: methyl *p*-coumarate; MCA: methyl caffeate; MSA: methyl sinapate; DSWB: destarched wheat bran.

From these results, and according to the putative classification of feruloyl esterases proposed previously [24], Ultraflo L contains predominantly a type B feruloyl esterase (active on MCA), with a small amount of type A (active on MSA). This result is in agreement with previous work [25], although assays were performed at lower temperature (37 °C), so the activity calculated was lower. Results were similar for the other two commercial preparations showing that the three commercial preparations contain the same types of FAEs, with Depol 740L exhibiting relatively higher activity on the hydrolysis of MSA possibly due to higher concentration of type A FAEs. According to the results obtained using DSWB as substrate (Table 1), it is concluded that Ultraflo L and Depol 740L contain almost the same total FAE activity, approximately 40% more than Depol 670L.

The three commercial enzyme preparations mainly showed xylanase activity while Depol 670L exhibited also polygalacturonase activity compared to the other three enzyme preparations (Table 2). FAE activity is low compared to the main chain degrading enzymes such as xylanase and β-glucanase due to the nature of these accessory acting enzymes which are expressed in low levels in the culture supernatant of the source microorganisms.

3.2. Immobilization studies

3.2.1. Effect of precipitant type on retention of FAE activity

As it is possible to form aggregates either by changing the hydration state of enzyme molecules or by altering the electrostatic constant of the solution adding the appropriate aggregation agents, it is crucial to select a suitable agent for aggregating the protein at the first step of CLEAs preparation process.

In this study 10 different types of precipitants were tested in order to determine their effect on enzyme activity recovery just after the precipitation and prior the cross-linking step. Results appear in Table 3 shows that saturated $(NH_4)_2SO_4$ and *t*-butanol were found to be the best precipitants and used in subsequent experiments. These agents were used as precipitants in many reports for the preparation of CLEAs [13,14,17–19] with ammonium sulfate to be more advantageous due to the low cost and its property to drop the temperature when it mixes with aqueous solutions. The latter is very important compared to the heat release produced by adding organic solvents in water as aggregation agents, especially in large-scale preparation of CLEAs

Table 2
Measurement of endoglucanase, xylanase, β-glucanase, arabinanase, polygalacturonase and FAE activities of the free and CLEAs form of the three commercial enzyme preparations

	Ultraflo L			Depol 670L			Depol 740L		
	U_{free}	U_{CLEAs}	% activity	U_{free}	U_{CLEAs}	% activity	U_{free}	U_{CLEAs}	% activity
Endoglucanase	1.35 (0.06)	1.01 (0)	74.70 (3.54)	9.70 (0.14)	11.30 (0)	116.30 (1.67)	0.75 (0.18)	0.08 (0)	11.00 (2.75)
Xylanase	70.07 (3.72)	34.30 (2.02)	49.00 (3.90)	31.00 (4.34)	31.64 (0.11)	102.20 (14.33)	47.70 (9.96)	21.30 (0)	44.70 (9.34)
β-Glucanase	1.67 (0.05)	2.02 (0)	121.30 (3.90)	6.00 (0.30)	2.26 (0)	37.47 (1.85)	5.10 (0.75)	0.82 (0)	16.20 (2.43)
Arabinanase	0.52 (0.02)	1.00 (0)	194.50 (9.20)	1.00 (0.01)	2.26 (0)	118.40 (6.90)	0.39 (0.057)	0.33 (0.08)	83.50 (24.10)
Polygalacturonase	Traces	0.10 (0)	–	13.50 (2.02)	6.78 (0)	50.40 (7.57)	0.13 (0.04)	0.08 (0)	62.60 (17.95)
FAE	0.06 (0)	0.10 (0)	171.80 (5.30)	0.03 (0.0)	0.11 (0)	313.90 (4.36)	0.06 (0.0)	0.08 (0)	146.20 (3.12)

The values corresponding to the activities of the free enzyme preparations (U_{free}) represent the total units present in 100 μL (which is the quantity used for the CLEAs preparative preparation). The activities of CLEAs (U_{CLEAs}) represent the total units of CLEAs powder (after freeze drying). The percentage activities of CLEAs (% activity) were calculated by taking the initial activities of the free enzymes as 100%. FAE activity was measured using DSWB as substrate. Assays were performed in duplicate and standard error is presented (number in parenthesis).

Table 3
FAE recovery of Ultraflo L, Depol 740L and Depol 670L after precipitation with different aggregation agents

Aggregation agent	Retention of initial activity (%)		
	Ultraflo L	Depol 740L	Depol 670L
Buffer MOPS 100 mM	100.0	100.0	100.0
Methanol	38.2 (0.1)	11.5 (0)	16.0 (0.1)
Ethanol	49.0 (0.1)	43.5 (0.4)	42.6 (0)
1-Propanol	45.7 (0.1)	30.4 (0.1)	20.0 (0.4)
2-Propanol	56.8 (4.4)	57.0 (0.3)	61.3 (0.7)
tert-Butanol	83.7 (0.3)	63.2 (0.6)	95.7 (0.8)
Acetone	80.7 (0.5)	62.8 (0)	29.0 (0.4)
Acetonitrile	72.9 (0.5)	66.8 (0.3)	71.6 (0.4)
Saturated $(NH_4)_2SO_4$ (767 g/L)	98.4 (0.1)	73.2 (0.5)	97.5 (0)
DMF	6.1 (0)	n.d.	n.d.
DMSO	18.6 (0.1)	53.9 (0.5)	n.d.

Assays were performed in duplicate and standard error is presented (number in parenthesis). n.d.: activity not detected; DMF: *N*,*N*-dimethylformamide; DMSO: dimethyl sulfoxide.

where the temperature is not properly controlled. Among the aliphatic alcohols tested, methanol caused almost totally loss of activity. Increase of alcohol's aliphatic chain resulted in higher retention of enzyme activity, with *t*-butanol being the best precipitant among the alcohols tested.

3.2.2. *Effect of glutaraldehyde concentration on immobilization efficiency*

Four different concentrations of glutaraldehyde (cross-linker) were employed using the saturated $(NH_4)_2SO_4$ or *t*-butanol as precipitants. Results appear in Fig. 1 show that the highest CLEA activities for Depol 740L and 670L were obtained with saturated $(NH_4)_2SO_4$ and 100 mM glutaraldehyde while for Ultraflo L with saturated $(NH_4)_2SO_4$ and 10 mM glutaraldehyde. These conditions were employed in subsequent experiments.

In principle, the precipitant with the highest recovery of activity is the best starting point for cross-linking. However, aggregate activity might differ from the redissolved enzyme, making it necessary to use multiple precipitants and assay the activity of the resulting CLEAs [19]. In the present study, *t*-butanol precipitation showed very good enzyme recovery activities for the three commercial preparations, making this precipitant a good candidate for the cross-linking step. However, cross-linking of the enzyme aggregates resulted in lower enzymatic activity of CLEAs or even complete inactivation in case of Depol 670L, revealing that the activity of the corresponding aggregates is decreased or completely diminished compared to the aggregates formed by the $(NH_4)_2SO_4$ precipitation. Under the employed optimum conditions (aggregate agent $(NH_4)_2SO_4$ and cross-linker 100 mM glutaraldehyde for Depol 740L and 670L and 10 mM for Ultraflo L) and using DSWB as substrate for assaying FAE activity, Ultraflo L, Depol 740L and Depol 670L exhibited significant activity (Table 2). Interestingly, in some cases, CLEA activity yields exceed that of the free enzyme activities exhibiting in the preparations before cross-linking. This hyperactivation is thought to find its origin in conformational changes of the protein induced by the aggregated state [15,19]. The immobilized forms of the aforementioned enzymes exhibit all the enzy-

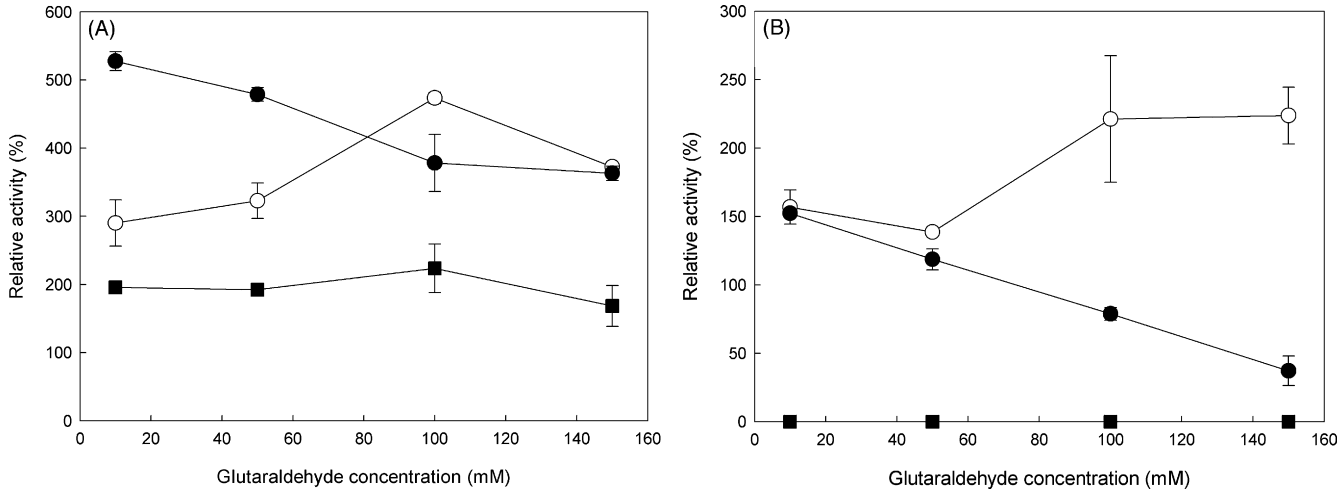

Fig. 1. FAE activity of the Ultraflo L (●), Depol 740L (◯) and Depol 670L (■) CLEAs, using (A) saturated $(NH_4)_2SO_4$ and (B) *t*-butanol as precipitant, for increasing glutaraldehyde concentration.

matic activities found in their initial commercial preparations (Table 2). Therefore, using the simple CLEA methodology, we succeeded in the simultaneous precipitation and cross-linking of different enzymatic activities present in the three commercial preparations, broadening the advantages of a carrier-free immobilized enzyme into a multipurpose biocatalyst.

3.3. Synthesis of 1-butyl ferulate

The synthetic activity of the three commercial enzymes was monitored by their ability to catalyse the transesterification reaction between MFA and 1-butanol in detergentless microemulsions. The synthetic performance of free enzymes was too low compared to the immobilized forms. In particular, 3.6% and 2.6% conversion was achieved by Ultraflo L and Depol 740L, respectively, while traces of product detected when Depol 670L was employed, after 6 days of incubation. The observed low reaction yields can be attributed to the precipitation which intervened with the addition of the free enzymes in the microemulsion reaction medium. In contrast, when transesterification performed by CLEAs (Fig. 2), conversion yields of 97%, 87% and 5% were obtained for Ultraflo L, Depol 740L and Depol 670L CLEAs, respectively. The conversion yields in case of Ultraflo L and Depol 740L CLEAs were significantly higher compared to the production of 1-butyl ferulate using as biocatalysts FoFaeB from *F. oxysporum* (12% [3]) or StFaeB (8% [26]) and StFaeC (20% [6]) from *S. thermophile*. The lower FAE activity present in Depol 670L preparation compared to the other two biocatalysts in combination with the possible instability of the FAEs in the organic reaction medium might be the reason for the low synthetic ability shown by the corresponding CLEAs.

3.4. Recovery and stability of CLEAs

One major advantage of CLEAs is their facile separation from the reaction medium. In contrast to free enzyme, brief centrifugation results in complete recovery of the biocatalyst. CLEAs of the three enzyme preparations were recycled by removing them from the reaction medium after 6 days of incubation and tested for their residual FAE activity. The recovered enzymatic activity for Ultraflo L, Depol 740L and Depol 670L was 48%, 36% and 35%, respectively, allowing the reuse of the enzymes for a second synthetic round. However, the performance of the recovered CLEAs during the second synthetic round was lower comparing to the first round (33% for Ultraflo L, 25% Depol 740L, traces for Depol 670L). After the end of the reaction there was no remaining FAE activity.

3.5. Structure of CLEAs

The control of the enzyme particle size in the CLEA process and its effect on the synthetic activity has rarely been reported. The particle size of the enzymes can influence mass transfer of the substrates to contact with the inner enzymes of the CLEAs [18]. If the particle size of the immobilized enzymes is too big, the inner enzymes of the CLEAs lose the opportunity to react

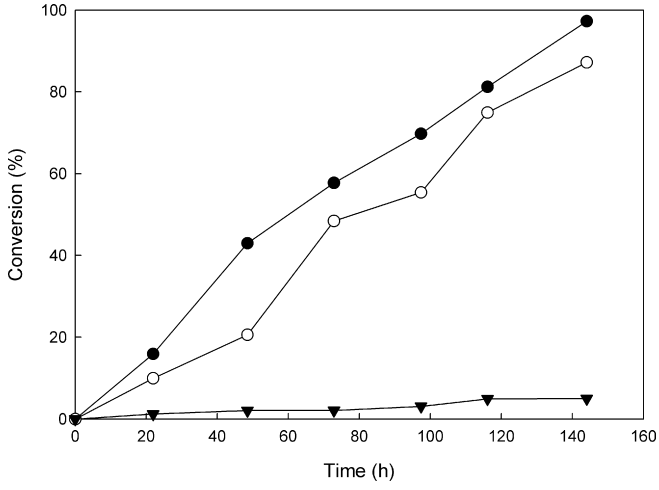

Fig. 2. Reaction progress for the transesterification of MFA with 1-butanol catalysed by 3.41 mU Ultraflo L (●), 2.98 mU Depol 740L (◯) and 1.91 mU Depol 670L (▼) CLEAs. The reactions were performed in *n*-hexane/1-butanol/water (47.2:50.8:2.0, v/v/v) ternary system, 40 mM MFA, 37 °C for 6 days at 1400 rpm.

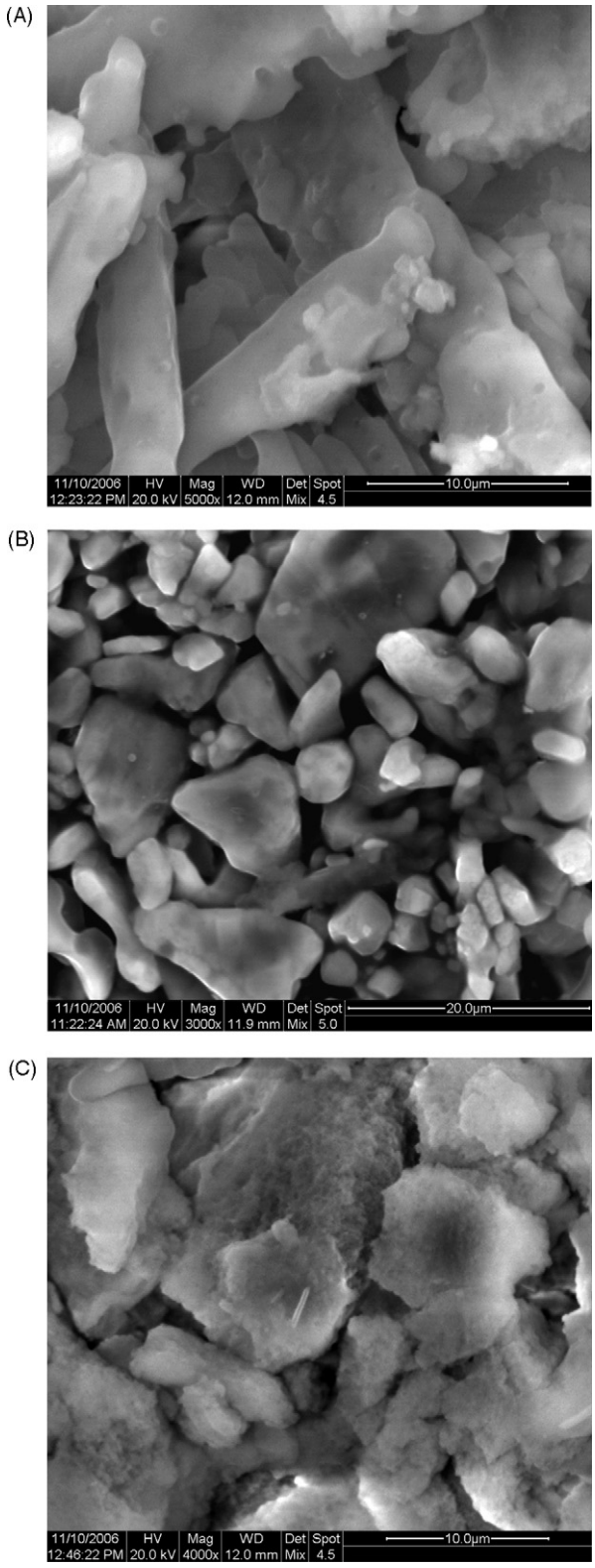

Fig. 3. SEM photographs of (A) Ultraflo L, (B) Depol 740L and (C) Depol 670L CLEAs at 5000×, 3000× and 4000× magnification, respectively.

with the substrates, wasting their activity. If the particle size of the enzymes is too small, all enzyme particles have a direct contact with organic solvents which could be harmful for their activity.

SEM of the three enzyme preparation CLEAs showed Type 2 structure of aggregates according to the work of Schoevaart et al. [19] (Fig. 3). While Type 1 aggregate forms typical 'balls', Type 2 aggregate clusters into less-defined structures. These aggregates are similar to *Candida rugosa* lipase and *Prunus amygdalus* R-oxynitrilase [19] which are glycosylated and therefore have a more hydrophilic surface. Since the enzyme molecules are packed inside relatively big aggregates, the CLEAs were finely dispersed in the reaction mixture using an Eppendorf Thermomixer.

4. Conclusions

An optimized procedure affords multipurpose immobilized biocatalyst for each of the three commercial fungal enzyme preparations which were found capable to catalyse the transesterification of ferulic acid into 1-butyl ferulate. The good catalytic activities and stability of CLEAs showing up to 97% conversion yield, easy recovery and reuse of the biocatalyst, opens an attractive and economic way for the modification of ferulic acid using carrier-free immobilized enzymes from cheap commercial enzyme preparations. Such multi-enzyme CLEAs are considered as useful preparations from both a laboratory and industrial point of view which can be used for different biotransformations in either aqueous or non-aqueous media.

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