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Journal of Molecular Catalysis B: Enzymatic 44 (2007) 8–13
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Esterification and transesterification reactions catalysed by addition of fermented solids to organic reaction media

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Received 16 May 2006; received in revised form 27 July 2006; accepted 14 August 2006
Available online 14 September 2006

Abstract

We report for the first time both the production of the lipase of *Burkholderia cepacia* in solid-state fermentation and the biocatalysis of esterification and transesterification reactions through the direct addition of the lyophilised fermented solids to organic reaction media. *B. cepacia* produced a lipolytic activity equivalent to 108 U of *p*NPP-hydrolysing activity per gram of dry solids after 72 h growth on corn bran with 5% (v/w) commercial corn oil as the inducer. The fermented solid material was lyophilised and added directly to the reaction medium in esterification and transesterification reactions. A factorial design was used to study the effects on esterification of temperature, alcohol-to-acid molar ratio and amount of lipolytic activity added. All three variables affected the ester yield significantly, with the amount of enzyme being most important. A 94% ester yield was obtained at 18 h at 37 °C, with an alcohol-to-acid molar ratio of 5:1 and 60 U of added lipolytic activity. For the transesterification reaction, a factorial design was undertaken with the variables being the alcohol-to-acid molar ratio and the added lipolytic activity. Ester yields of over 95% were obtained after 120 h. Our results suggest that biocatalysis using direct addition of fermented solids to organic reaction medium should be further explored.

Keywords: Biocatalysis; Solid-state fermentation; SSF; Lipase; *Burkholderia cepacia*

1. Introduction

Esterification and transesterification are industrially important reactions. Amongst other applications, esterification is important in the production of flavours and aromas while transesterification is currently of great interest for the production of biodiesel esters. These reactions are typically carried out chemically in industry, however, the chemical routes have several disadvantages. For example, the chemical route involves either acid or base catalysis, and any residues of acid or base in the product are undesirable. Further, the chemical route involves temperatures above ambient, which not only lead to high consumption of energy, but also can lead to degradation of the ester product [1].

Lipases (E.C. 3.1.1.3) can catalyse esterification and transesterification reactions in organic media, and therefore production of esters by a biocatalytic route presents itself as an attractive alternative. The use of lipases brings the advantages of high selectivity, high specificity and mild operating conditions [1,2]. In fact, lipases are already being used to catalyze the synthesis of esters and the transesterification of oils to produce biodiesel esters [1–4].

To date the traditional approach in the biocatalytic route for esterification and transesterification has been to use commercial microbial lipases produced in submerged fermentation. Typically it is advantageous to immobilize the enzyme on solid

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doi:10.1016/j.molcatb.2006.08.004

supports so that they are easily recovered and reused. This was the case in studies of biodiesel production with the commercial lipases of *Candida antarctica* (Novozyme 435), *Candida rugosa* (Amano), *Chromobacterium viscosum*, *Rizhomucor miehei*, *Pseudomonas cepacia* (Amano) and *Pseudomonas fluorescens* (Amano) [5–9]. However, this approach is expensive because it requires both the concentration of the culture broth to produce a crude extract and immobilization of the enzyme.

In this context, production of the lipase by solid-state fermentation (SSF) presents itself as an interesting possibility. In SSF systems the organism grows on particles of a moist solid organic material, with a minimum of free water in the spaces between the particles. The fermented solid can act as a support for the enzyme, without the need for a previous extraction step, as shown by Nagy et al. [10], who used direct addition of dried fermented solids to catalyse the enantiomeric resolution of racemic secondary alcohols.

In the current work we demonstrate the feasibility of producing a lipase from *Burkholderia cepacia* in SSF and using the fermented solid to catalyse esterification and transesterification reactions, using the production of ethyl-oleate and the transesterification of corn oil with ethanol as model systems.

2. Materials and methods

2.1. Production of lipases by solid-state fermentation

A strain of *B. cepacia* showing excellent halo-forming ability on agar plates that contained, per litre of distilled water, 15 g agar, 10 mL olive oil, 0.01 g Rhodamine B and 0.001% (w/v) Tween 80 was selected and identified by IRD (ex-ORSTOM), Laboratoire de Microbiologie, Université de Provence, CESB/ESIL, Marseille, France. It was freeze-dried in glass ampoules and maintained at −20 °C. For the seed culture, it was inoculated into 50 mL of Luria Bertani broth in a 125 mL Erlenmeyer flask and incubated at 29 °C and 120 rpm. After 8 h, at which time it was in mid-exponential phase, it was harvested and diluted with distilled water to give 1.0×10^8 cells/mL.

Corn bran was kindly donated by Corn Products Brazil, São Paulo, Brazil. The fraction between (0.8 and 2.0 mm) was dried at 60 °C. Each 250 mL Erlenmeyer flask contained 10 g of this dried meal, 0–5% (v/w) corn bran oil and 0.1 mol/L pH 7.0 phosphate buffer, added to adjust the moisture content to 55% (w/w, wet basis). Flasks were autoclaved at 121 °C for 15 min. Each flask was then inoculated with 1 mL of diluted seed culture and incubated at 29 °C.

2.2. Construction of the fermentation profile in terms of lipolytic activity

Triplicate flasks were processed for each treatment at each sampling time. Each flask received 50 mL of 2% (w/v) NaCl and was then agitated for 1 h at 200 rpm at 29 °C. The suspension was filtered through cheesecloth, the excess liquid being squeezed out manually. The extract was centrifuged for 10 min at 10,000 × *g* and the protein concentration and lipolytic activity of the supernatant, hereafter referred to as the aqueous extract, were then determined.

2.3. Lipase activity measurement

Two methods were used to determine the lipolytic activity in the aqueous extract: the spectrophotometric method of hydrolysis of *p*-nitrophenyl palmitate (*p*NPP, Sigma) [11] and the titrimetric method, using tributyrin (C_4), tricarpylin (C_8) and triolein (C_{18}) as substrates (Sigma) [12]. For both methods the activity (U) was referred to the fermented dry solid in units of activity per gram of dry solids (U/gDS).

For the *p*NPP method, the coefficient of extinction of *p*-nitrophenol (*p*NP) at pH 7.0 was determined as 8×10^3 L mol^{-1} cm^{-1} at 410 nm. One unit of activity (U) was defined as the production of 1 μmol of *p*-nitrophenol per minute at 37 °C and pH 7.0, under the conditions of the assay.

For the titrimetric method, emulsions of the substrates were prepared and the free fatty acids were titrated using 0.05 M NaOH in a pH-stat (Metrohm 718 Stat Titrino) set at a constant pH value. Each assay was performed in a thermostated vessel (25 °C) at pH 8.0. For tributyrin and tricaprylin, the reaction medium contained 0.25 mM Tris–HCl buffer and 150 mM NaCl [13], plus either 63 mM of tricaprylin or 54 mM of tributyrin. When triolein was used, the substrate (62 mM) was pre-emulsified with gum Arabic (3%, w/v) and $CaCl_2$ (2 mM) [14]. The rate of spontaneous substrate hydrolysis was recorded for 2 min prior to lipase injection. One unit of enzyme activity was defined as the liberation of 1 μmol of fatty acid per minute, under the assay conditions.

2.4. Lyophilisation of fermented solid for biocatalysis

After 72 h incubation, fermented solid prepared as described in Section 2.1 was lyophilised for 12 h at −45 °C in a Jouan LP3 Lyophilizer and then stored at 4 °C. The loss of activity due to the lyophilisation was 10% (based on *p*NPP-hydrolysing activity). This material was used directly in ester synthesis.

2.5. Delipidation of the fermented solid

In some cases the fermented solid was delipidated after lyophilisation to remove monoolein, diolein, free fatty acids and residual triglycerides deriving from the fermentation. A 30 mL aliquot of a chloroform:butanol (9:1) mixture was added to 2 g of fermented material and the mixture was agitated vigorously for 10 min at 200 rpm. The solution was then filtered through cheesecloth, the excess liquid being squeezed out manually. The entire procedure was carried out at 25 °C. The filtrate was analysed by TLC (thin-layer chromatography). Triolein and oleic acid were used as standards. The delipidation was repeated until no lipids were detected on the TLC plate.

2.6. Optimization of the synthesis of ethyl-oleate

The standard assay of synthesis of ethyl-oleate was done in 25 mL Erlenmeyer flasks in a shaker at 200 rpm, using 5 mL

of organic medium with *n*-heptane as the solvent and sufficient lyophilized corn bran to give 30 U of activity according to the *p*NPP method. The reaction was started by the addition of ethanol and oleic acid, the latter always starting at 70 mmol L^{-1}. At fixed intervals, 100 μL samples of the mixture were collected and analyzed for residual free fatty acids by the Lowry–Tinsley method, which has proved to be reliable in previous work [3,15]. A standard curve was constructed using oleic acid under the same conditions. The ester yield was calculated from the consumption of free fatty acids. Two controls were done: one containing the solid material prepared as described in Section 2.1, but without inoculation of *B. cepacia* and one containing autoclaved fermented solid. In both cases there was no ester production, showing that the conversion was due to the lipolytic enzymes produced during the fermentation.

In the study of the necessity for delipidation, the molar ratio of ethanol to oleic acid was 3. The reaction was undertaken at 37 °C. In the factorially designed experiment, lyophilised fermented non-delipidated corn bran was used, under the conditions described in Table 1.

2.7. Transesterification reactions

A 2^2 factorially designed experiment was carried out with the variables being the alcohol-to-corn-oil molar ratio and the amount of lipolytic activity added to the reaction mixture. Reactions were done in *n*-heptane on an orbital shaker at 150 rpm and 37 °C for 120 h. At 120 h the reaction medium was filtered, evaporated and then dissolved in an appropriate solvent. The ethyl-esters (of linolenate, linoleate, oleate, estearate and palmitate) were analyzed by high-performance liquid chromatography (HPLC). The ester yield (Y) was calculated as

$$Y = \frac{M_W T_E}{M_O F_C} \times 100\% \quad (1)$$

where T_E is the content of esters and F_C converts the original mass of oil to a theoretical mass of ethyl-ester products based on 100% conversion of the fatty acids in the oil. The value of F_C for corn oil (1.053) was calculated on the basis of a weighted average that took into account the fatty acid composition of corn oil [16].

2.8. Analytical methods

Analytical TLC was performed on silica gel 60 plates containing fluorescein (Merck) using hexane/ether/acetic acid 7:3:0.1 (v/v/v) as eluent. Esters and free fatty acids were visualized in UV chamber (254 nm) after spraying the plate with iodine.

HPLC was done in a Shimadzu Model LC 10 AD chromatograph equipped with a Waters Spherisorb (4.6 mm × 250 mm, 5 (m) C_{18} column and a refraction index detector (RID 10 A), with integration being done using the CLASS 10 software (Shimadzu). For the analysis of ethyl-esters, a 20 μL aliquot of the sample was injected and eluted isocratically with acetonitrile/acetone 9:1 (v/v) at 0.9 mL/min and 35 °C. Ester contents were quantified based on a calibration curve constructed using external standards of ethyl-esters of linolenic, oleic, palmitic and stearic acids.

2.9. Statistical analysis

In the factorial experiments the contrast coefficients (C_i) were calculated as

$$C_i = \frac{\sum EL_i + \sum EH_i}{4} \quad (2)$$

where C_i is the contrast coefficient of factor i and E is the percentage conversion of oleic acid to ester. L_i and H_i are the lower and higher levels of parameter i, respectively.

3. Results

3.1. Production of lipase on corn bran

Corn bran, a by-product of the extraction of corn oil, contains 17% protein and 2% oil (% by mass on a dry basis). It is produced in large amounts (about 150 tonnes per day by Corn Products Brazil, Mogi das Cruzes, São Paulo) and is normally incorporated into pet food. Since its lipid content is relatively low, we investigated the influence of adding commercial corn oil to the corn bran. Note that initial corn oil concentrations above 5% (v/w) were not investigated because they conferred a very sticky consistency on the corn bran solids, making them unsuitable for SSF.

The maximum *p*NPP-hydrolysing activity, 108 U/gDS, was obtained when 5% (v/w) corn oil was added to the corn bran (Fig. 1). The corresponding activity determined by the titrimetric method was 84 U/gDS for tributyrin, 76 U/gDS for tricaprylin, and 42 U/gDS for triolein, confirming that true lipolytic activity was present. These results are promising because they show that an industrial residue can be used to produce the lipase of *B.*

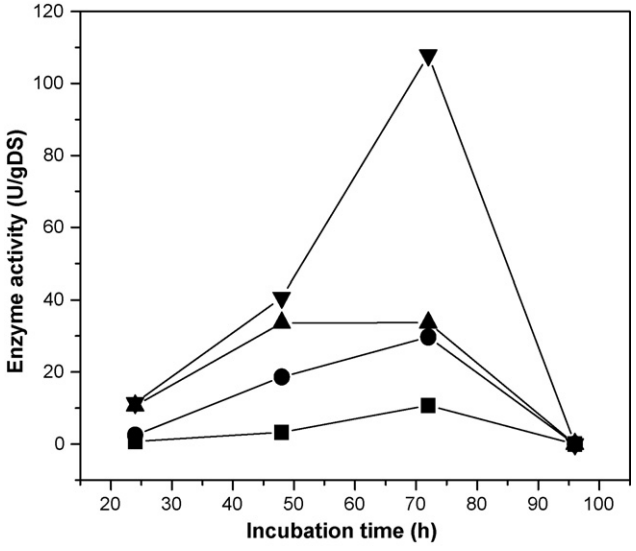

Fig. 1. Effect of supplementation with corn oil on the production of lipase at 29 °C by *Burkholderia cepacia* in solid-state fermentation of corn bran. Corn oil levels: (■) 0%; (●) 1%; (▲) 3%; (▼) 5% (v/w).

Table 1
Experimental conditions and results of a 2^3 factorial design to optimize the production of ethyl-oleate using the fermented solid containing a lipase produced by *Burkholderia cepacia* in solid-state fermentation

Run	Temperature (°C)	Total units (U)	M[a] (alcohol:acid)	Yield after 18 h (% of the theoretical maximum)
1	30	90	7.0	64
2	30	30	7.0	70
3	43	90	3.0	51
4	43	30	3.0	21
5[b]	37	60	5.0	94
6	43	90	7.0	82
7	30	30	3.0	50
8	43	30	7.0	41
9	30	90	3.0	69

[a] M is the alcohol-to-acid molar ratio. In each case the oleic acid concentration was 70 mM.
[b] Run 5 was done in triplicate and allowed the calculation of the standard deviation ±1.67

cepacia, which is commonly used in organic synthesis [14]. To demonstrate the viability of using this fermented solid directly in biocatalysis, we used the production of ethyl-oleate and the transesterification of corn oil with ethanol as model systems.

3.2. Optimisation of ester synthesis using the lipase produced on corn bran

Corn bran with an initial moisture content of 55% (w/w, wet basis) and 5% (v/w) corn oil was fermented at 29 °C for 72 h, at which time the lipase activity was 120 U/gDS. The fermented solid was then lyophilised. In order to determine the effect of the lipids present in the fermented material on the reaction rates, we performed two preliminary experiments: direct addition of delipidated and non-delipidated lyophilised solids. In both cases the final activity of the solids was 108 U/gDS. Ester yields of 100 and 97.4% were obtained after 24 h for the delipidated and non-delipidated material, respectively (Fig. 2), showing that there is no need to delipidate the enzyme before using it in ethyl-oleate synthesis. Non-delipidated solids were used in all the remaining studies.

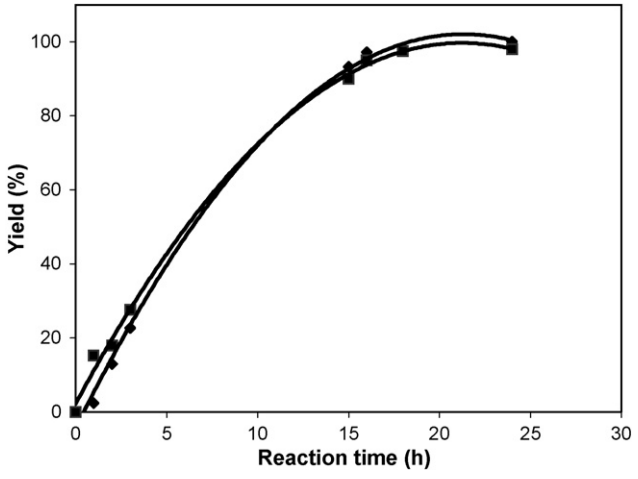

Fig. 2. Kinetics of ethyl-oleate synthesis using delipidated (■) and non-delipidated (●) fermented solids. Reactions were carried out at 37 °C in *n*-heptane with a molar ratio of ethanol to oleic acid of 3.0 and sufficient solids to provide 30 U of lipolytic activity.

For comparative purposes, an aqueous extract, prepared as described in Section 2.3 and lyophilized as described in Section 2.4, was added directly to the reaction mixture and incubated under the same conditions. After 25 h the ester yield was only 42%, even when 60 U of lipolytic activity was added.

A 2^3 fractional factorial design was used to determine the effects of temperature, molar ratio of alcohol to acid and the amount of enzyme on the reaction. The kinetics of each reaction were followed for 24 h. Table 1 compares ester yields at 18 h, the time at which the best reaction conditions reached a yield of over 90%.

The contrast coefficients (C_i) calculated from these results are shown in Table 2. All individual and combined variables affected the ester yield significantly, since all C_i values were greater than the standard deviation obtained for the triplicate of the central point (run 5). The most significant individual effect was the amount of added enzyme (U), with a C_i of +22. This means that increasing the enzyme level from 30 to 90 U leads to a 22 percentage point (p.p.) increase in the reaction yield. However, with high enzyme levels it became difficult to agitate the reaction medium due to the high solids concentration. Increasing the alcohol-to-acid molar ratio from 3:1 to 7:1 increased the ester yield by 17 p.p. (C_i of +17), while increasing the temperature from 30 to 43 °C decreased the ester yield by 14 p.p. ($C_i = -14$).

All interaction effects were also important. The interaction between the temperature and the alcohol-to-acid molar ratio was most significant, with a C_i of −46. The next most important

Table 2
Contrast coefficients (C_i) calculated from the 2^3 factorial design of Table 1

Variables	C_i
T (°C)	−14
Total units (U)	+22
M (alcohol-to-acid)	+17
$T \times U$	+14
$T \times M$	−46
$U \times M$	−3
$T \times U \times M$	+9

M is the alcohol-to-acid molar ratio, T the temperature and U is the units of lipase activity. The standard deviation calculated from a triplicate of run 5 (Table 1) was ±2.

Table 3
Experimental conditions and results of a 2^2 factorial design to study the transesterification of corn oil using the fermented solid containing a lipase produced by *B. cepacia* in solid-state fermentation

Experiment	*M*[a] (alcohol:oil)	Total units (U)	Ester yield[b] (%)
1	3.0	16.9	83.5
2	3.0	67.6	94.2
3	6.0	16.9	85.4
4	6.0	67.6	94.7
5[c]	4.5	43.9	93.1

[a] *M* is the alcohol-to-oil molar ratio. In each case the corn oil concentration was 70 mM.
[b] Ester yields determined after 120 h of reaction.
[c] Run 5 was done in triplicate and allowed the calculation of standard deviation of ±0.1.

Table 4
Contrast coefficients (C_i) calculated from the factorial design 2^2 of Table 3

Variables	C_i
Total units (U)	+10.5
M (alcohol-to-oil)	+1.8
$U \times M$	−1.2

M is the alcohol-to-oil molar ratio and *U* is the units of lipase activity. The standard deviation calculated from a triplicate of run 5 (Table 3) was ±0.1.

interaction was that between the temperature and the amount of enzyme, with a C_i of +14. The complexity of the system is shown by the fact that the tertiary interaction ($T \times U \times M$) was also significant ($C_i = +9$).

The best conditions for the synthesis of ethyl-oleate were obtained at the central point (37 °C, a molar ratio of ethanol to oleic acid of 5.0 and 60 U of lipolytic activity added), at which 94% of the acid was converted after 18 h.

3.3. Transesterification using the lipase produced on corn bran

In order to study the conditions of the transesterification reaction, a 2^2 factorial experiment was undertaken, with the variables being the molar ratio of ethanol to oil and the lipolytic activity added. On the basis of the results of the esterification experiment, the reaction was carried out at 37 °C. The ester yields ranged from 83.5 to 94.7% (Table 3). All individual and combined variables affected the ester yield significantly, since all C_i values were greater than the standard deviation of run 5 (±0.1) (Table 4). The most significant variable affecting yields was the amount of lipase activity added ($C_i = +10.5$), followed by the ratio alcohol-to-oil (*M*) and the interaction between these two variables ($C_i = -1.2$).

4. Discussion

This paper makes two important contributions. Firstly, we have shown that the lipase of *B. cepacia* can be produced in SSF. Secondly, we have shown that the fermented solid material can be added directly to an organic medium in order to catalyze ester synthesis and transesterification reactions. These contributions are discussed separately below.

4.1. Significance of the production of B. cepacia lipase in SSF

SSF is often claimed to be most suitable for processes involving filamentous fungi, due to the low water availability in this system. In fact, although bacteria have been used to produce protease, α-amylase and xylanase in SSF [17–22], the great majority of SSF processes for enzyme production involve fungi and yeasts [23]. This is especially true for lipase production in SSF: processes have previously been reported for the production of fungal and yeast lipases, but there are few previous reports of the production of bacterial lipases [22]. The production of the lipase of *B. cepacia* in SSF is highly promising since this enzyme finds many applications in biocatalysis due to its stability in organic solvents, its high selectivity when used for the resolution of racemic secondary alcohols and its ability to catalyze enantioselective synthesis [10]. Production of this enzyme in SSF using agro-industrial residues can potentially lower production costs significantly; projected costs (per unit of activity) for lipase production by SSF of residues are three-fold lower than for a traditional submerged fermentation process [24].

4.2. Significance of the direct application of fermented solid in biocatalysis

We have shown for the first time that fermented solids can be used directly to catalyze esterification and transesterification reactions. The only previous study of direct addition of fermented solids concerned itself with the lipase-catalysed kinetic resolution of racemic secondary alcohols [10], producing higher value products. If these products are to be as intermediates in pharmaceutical synthesis, it may in fact be preferable to use a relatively pure enzyme produced by submerged fermentation in order to minimize the risk of introducing contaminants: Direct addition of dried fermented solids to the reaction mixture might bring contaminating material from either the original solids or the organism. Such contaminants would be of less importance in the production of biodiesel ester or even in the biocatalytic production of food aromas using GRAS (generally recognized as safe) organisms and food processing by-products as fermentation feedstocks.

Our reaction rates in ester synthesis (94% in 18 h) were quite comparable with those obtained for ester synthesis by other authors in the so-called “traditional” biocatalytic systems. In the reversed micellar system, conversion times are often somewhat higher. For example, 168 h were required for a 95% yield of ethyl-caproate using an esterase produced by *Bacillus licheniformis* [25] while 120 h were required for 95–100% yield of butyl-palmitate using the lipase of *Candida cylindracea* [26]. However, shorter reaction times have also been reported. For example, 12 h was sufficient for a 100% yield of butyl-oleate using the lipase of *Penicillium coryophilum* [27].

Similarly, our reaction rate was slower than some literature results using immobilized enzymes, but faster than others.

For example, 72 h were required for an 86% yield of ethyl-hexanoate, using the lipase of *C. rugosa* immobilized on a polymer made from polyvinyl alcohol (PVA), alginate and boric acid [28]. On the other hand, 6 h were sufficient for a 100% yield of butyl-oleate using the lipase of *B. cepacia* immobilized on Accurel [29].

Our transesterification rates are lower than typical values from the literature. For example, 24 h was sufficient for a 97% yield of methyl-esters from methanol and soybean oil [30] while 48 h was sufficient for 94–96% methyl ester yields from degummed and refined soybean oil [31]. In both cases the lipase of *C. antarctica* was used as the biocatalyst, being produced by submerged fermentation and then immobilized. If we can improve our transesterification rates, then our biocatalyst will become highly competitive due to its low production costs. Note that it might be possible to reduce its costs even further, if the lyophilisation can be replaced with a mild air drying.

5. Conclusions

We have shown that the lipase of *B. cepacia* can be produced in solid-state fermentation using an industrial residue and also that the direct addition of a fermented solids containing lipolytic activity to an organic reaction system can give acceptable rates in the synthesis of ethyl-oleate. The transesterification of corn oil with ethanol is also possible, although longer reaction times are necessary. The system of adding the fermented solid to the reaction medium has two aspects that can potentially reduce the costs of biocatalysis with lipases significantly, which will be of great importance in the production of relatively low-value products such as biodiesel esters. Firstly, the feedstock for the production of the lipase is a waste product of corn processing and is available at minimal cost. Secondly, the direct use of the lyophilised fermented solid avoids the need for expensive enzyme recuperation and immobilization processes. The potential of this system in biocatalysis deserves to be explored further.

Acknowledgements

Research scholarships were granted to Luiz Ramos, David Mitchell and Nadia Krieger by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brasil) and to Alessandra Baron by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brasil). The work was supported financially by two grants administered by CNPq ("CT-AGRO" and "Universal"). We also wish to thank Dr. Bernard Ollivier and Dra. Marie-Laure Fardeau from IRD Laboratoire de Microbiologie, Université de Provence, CESB/ESIL, Marseille, France for the characterization of the bacterium and Corn Products Brazil for the donation of the corn bran.

References

[1] P. Villeneuve, J.M. Muderhwa, J. Graille, M.J. Haas, J. Mol. Catal. B: Enzym. 9 (2000) 113.
[2] S.H. Krishna, B. Manohar, S. Divakar, S.G. Prapulla, N.G. Karanth, J. Biotechnol. 87 (2001) 191.
[3] M.L.M. Fernandes, N. Krieger, A.O. Baron, P.P. Zamora, L.P. Ramos, D.A. Mitchell, J. Mol. Catal. B: Enzym. 30 (2004) 43.
[4] R. Sharma, Y. Chisti, U.C. Banerjee, Biotechnol. Adv. 19 (2001) 627.
[5] G. Trubiano, D. Borio, M.L. Ferreira, Biomacromolecules 5 (2004) 1832.
[6] S.M. Radzi, M. Basri, A.B. Salleh, A. Ariff, R. Mohammad, M.B.A. Rahman, R.N.Z.R.A. Rahman, Electron. J. Biotechnol. 8 (2005) 292.
[7] A. Salis, M. Pinna, M. Monduzzi, V. Solinas, J. Biotechnol. 119 (2005) 291.
[8] H. Ghamgui, M. Karra-Chaâbouni, Y. Gargouri, Enzyme Microb. Technol. 35 (2004) 355.
[9] S. Shah, S. Sharma, M.N. Gupta, Indian J. Biochem. Biophys. 40 (2003) 392.
[10] V. Nagy, E.R. Tòke, L.C. Keong, G. Szatzker, D. Ibrahim, I.C. Omar, G. Szakàcs, L. Poppe, J. Mol. Catal. B: Enzym. 39 (2006) 141.
[11] U.K. Winkler, M. Stuckmann, J. Bacteriol. 3 (1979) 663.
[12] W. Stuer, K.E. Jaeger, U.K. Winkler, J. Bacteriol. 168 (1986) 1070.
[13] J.C. Mateos Diaz, J.A. Rodríguez, S. Roussos, J. Cordova, A. Abousalham, F. Carrière, J.C. Baratti, Enzyme Microb. Technol. (2006).
[14] K.E. Jaeger, B.W. Dijkstra, M.T. Reetz, Annu. Rev. Microb. 53 (1999) 315.
[15] R.R. Lowry, J.I. Tinsley, J. Am. Oil Chem. Soc. 53 (1976) 470.
[16] F. Ma, M.A. Hanna, Bioresource Technol. 70 (1999) 1.
[17] R.S. Prakasham, C.S. Rao, R.S. Rao, P.N. Sarma, Biotechnol. Prog. 21 (2005) 1380.
[18] V.F. Soares, L.R. Castilho, E.P.S. Bom, Appl. Biochem. Biotechnol. 121 (2005) 311.
[19] H.K. Sodhi, K. Sharma, J.K. Gupta, Process Biochem. 40 (2005) 525.
[20] A.A. El-Bessoumy, M. Sarhan, J. Manssur, J. Biochem. Mol. Biol. 37 (2004) 387.
[21] H.M. Patel, R.H. Wang, O. Chandrashekar, Biotechnol. Prog. 20 (2004) 110.
[22] K.R. Babu, T. Satyanarayana, J. Sci. Ind. Res. 55 (1996) 464.
[23] A. Pandey, C.R. Soccol, D.A. Mitchell, Process Biochem. 35 (2000) 1153.
[24] L.R. Castilho, C.M.S. Polato, E.A. Baruque, G.L. Sant'Anna Jr., D.M.G. Freire, Biochem. Eng. J. 4 (2000) 239.
[25] E. Alvarez-Macarie, J.C. Baratti, J. Mol. Catal. B: Enzym. 10 (2000) 377.
[26] A.M. Rao, M.A. Murray, V.T. John, Biocatalysis 4 (1991) 253.
[27] A.M. Baron, M.I.M. Sarquis, M. Baigori, D.A. Mitchell, N. Krieger, J. Mol. Catal. B: Enzym. 34 (2005) 25.
[28] R. Dave, D. Madamwar, Process Biochem. 41 (2006) 951.
[29] A. Salis, E. Sanjust, V. Solinas, M. Monduzzi, J. Mol. Catal. B: Enzym. 24 (2003) 75.
[30] T. Samukawa, M. Kaieda, T. Matsumoto, K. Ban, A. Kondo, Y. Shimada, H. Noda, H. Fukuda, J. Biosci. Bioeng. 90 (2000) 180.
[31] Y. Watanabe, Y. Shimada, A. Sugihara, Y. Tominaga, J. Mol. Catal. B: Enzym. 17 (2002) 151.