Organic Process Research & Development **2002**, *6*, 441−445

High-Throughput Screening of Thermostable Esterases for Industrial Bioconversions

Delphine Lagarde,[†] Hong-Khanh Nguyen,[†] Gilles Ravot,[†] Denis Wahler,[‡] Jean-Louis Reymond,[‡] Geoffrey Hills, Thomas Veit,[§] and Fabrice Lefevre*[,†,‡,§]

Proteus SA, 70 allée Graham Bell, 30000 Nîmes, France, University of Bern, Department of Chemistry and Biochemistry, Freiestrasse 3, 3012 Bern, Switzerland, and Degussa AG, Goldschmidtstrasse 100, 45127 Essen, Germany

Abstract:

Thermostable esterases are used in biocatalysis for the synthesis of a variety of products, for example, flavour esters, emulsifiers, or pharmaceutical intermediates. Industrial enzymatic conversions to obtain chemo-, regio-, and stereoselective transformations may require thermostable enzymes active in organic solvents at high temperatures. To find novel enzymes for industrial bioconversions, we have used a new generation of thermostable reporter substrates (CLIPS-O for CataLyst Identification ProcesS per Oxidization) to screen for esterase activity in thermophilic microorganisms belonging to various thermophilic genus. One of the strains (belonging to *Thermus* genus) exhibiting activity on short- and long-chain ester CLIPS-O substrates has been selected. The genomic library of this strain was screened using a high-throughput functional technology. Positive clones expressing esterases active at high temperature were immediately characterized during screening for chain-length specificity.

1. Introduction

The use of enzymes as biocatalysts to carry out chemo-, regio-, and stereoselective reactions on a wide range of natural and synthetic substances is becoming increasingly important, as is evident from the number of publications in this field. Esterases and lipases are an important group of enzymes which can be used to catalyze hydrolysis, esterification, and transesterification reactions to generate various acyl derivatives or alcohols. Typical potential applications are the synthesis of regioisomers of polyfunctional compounds, resolutions of secondary alcohols, and asymmetric syntheses from prochiral or meso compounds.[1−3]

Many examples in the literature describe the use of these enzymes for such bioconversions, but in some cases the activities and selectivities obtained are not satisfactory. It is clear that there are too few enzymes from the potentially vast pool existing in nature which are commercially available to investigate the feasibility of carrying out industrially interesting syntheses.

One way of altering the properties of existing enzymes, such as stereoselectivity or thermostability, is to change the protein structure by one or a few amino acids by mutagenesis techniques.[4] However, this can be laborious, and there is no guarantee of achieving a successful result.

Another better way to obtain new enzymes which have the required properties to carry out a specific bioconversion is to screen the natural biodiversity. One group of microorganisms which has not been investigated thoroughly, but which has in the past few years received much attention, is that consisting of extremophiles.[5]

Such microorganisms, which can grow in harsh environments of high and low temperature or extreme pH and ionic strength or both, are equipped with enzymes having interesting characteristics of stability. Some of these enzymes may be valuable as biocatalysts in industrial processes, where the properties of enzymes must fit processing conditions since it is not always possible to adapt a process to meet the optimum functional activity range of enzymes. For example, a certain pH or temperature of a solution containing substrates or products may be necessary for production purposes, which may not be optimal for the activity and stability of the enzyme.

One area of importance to improve industrial biocatalysis is to perform reactions at higher temperatures, which generally results in faster reaction rates (as in conventional chemical catalysis) and in particular cases could open up routes to the economical production of new substances. For example, organic solvents are often needed to solubilize reactants and products. If reactions can be performed in molten raw materials at higher temperatures, the use of toxic solvents and the added expense of their removal from products can be avoided. The development of such environmentally friendly processes is an important aspect of biotechnology. As many enzymes are often unstable above normal physiological temperatures or growth conditions, the screening for thermostable enzymes from microorganisms adapted to living in extreme environments is thus an important way to find the right biocatalysts for desired reactions.

* Author to whom correspondence should be sent. E-mail: flefevre@proteus.fr, www.proteus.fr. Telephone: +33 4 66 70 64 64. Fax: +33 4 66 70 64 60.

[†] Proteus SA.

[‡] University of Bern.

[§] Degussa AG.

(1) Bornscheuer, U. T., Kazlauskas, R. J. *Hydrolases in Organic Synthesis: Regio- and Stereoselective Biotransformations*; Wiley-VCH: Weinheim, 1999.

(2) Liese A. *Industrial Biotransformations*; Wiley-VCH: Weinheim, 2000.

(3) Schmidt, R. D.; Verger, R. *Angew. Chem.* **1998**, *110*, 1694−1720.

(4) Jaeger, K.-E.; Eggert, T.; Eipper, A.; Reetz, M. T. *Appl. Microbiol. Biotechnol*. **2001**, *55*, 519−530.

(5) Adams, M. W. W.; Kelly, R. M. *Chem. Eng. News* **1995**, *73*, 32−42.

10.1021/op020019h CCC: $22.00
Published on Web 06/27/2002

A number of thermostable esterases (or lipases) have already been reported from various microorganisms within the three domains of life: *Archaea*, *Bacteria*, and *Eucarya*.[6] Concerning prokaryotics, intracellular thermostable esterases have been reported from Archaeon including S*ulfolobus acidocaldarius*[7] and *Archaeoglobus fulgidus*.[8] Within the domain of *Bacteria*, intracellular esterases have also been characterized from many sources including *Bacillus* species.[9]

In parallel, extracellular esterases have been described from bacterial sources (*Bacillus subtilis*, *Streptomyces scabiei*, and *Clostridium stercorarium*) and from one *Archaea*: *Sulfolobus shibatae*.[10,11]

Industrial transformation of long-chain aliphatic acid esters requires that it be carried out at high temperature for solubility reasons, especially to avoid aggregation of substrate or product or both. Therefore, to keep the advantages of a biocatalytic process for such a transformation, new thermostable enzymes are needed, which would answer the problems linked to the conditions of the reaction.

Here, we report the screening, cloning, and characterization (in terms of carboxylic acid substrate specificity) of novel thermoactive esterases from a *Thermus* strain, using a new functional assay technology called CLIPS-O.

2. Materials and Methods

2.1. Strains. *Thermus* sp. P1074 is a strain from the exclusive biodiversity collection of microorganisms of Proteus. This strain was isolated from the nonvolcanic-heated water of the Australian Artesian Basin in the laboratory of Professor B. K. C. Patel (Griffith University, Australia).

The *Escherichia coli* MC1061 strain (F^- *araD139* Δ*(ara-leu)7696 galE15 galK16* Δ*(lac)X74 rpsL (Str^r) hsdR2* (r_k^- m_k+) *mcrA mcrB1*) was used as a recipient host for the genomic library amplification of the *Thermus* strain and for expression and activity assays of the correspondent thermophilic proteins (as described in ref 12[12]).

2.2. Culture Media and Growth Conditions. The *Thermus* strain P1074 was grown in medium D (as described in ref 13[13]) at 70 °C in shake flasks (180 rpm). Induction of esterase expression was achieved by addition of 2% (v/v) of olive oil to medium D.

E. coli MC1061 was grown in Luria−Bertani (LB) medium containing 100 mg/L of ampicillin at 37 °C in orbital shakers for 16 h.

2.3. Genomic DNA Library. Genomic DNA was isolated from 150 mL of culture using QIAamp DNA Mini Kit (Qiagen). Plasmid library was constructed by ligation of genomic DNA, which was previously partially digested by *Sau*3AI, into the *Bam*HI site of a specific proprietary vector called pBANK. The size of cloned inserts ranged from 2 to 4 kb. The plasmid library was amplified by transformation of *E. coli* MC1061 strain. Isolated *E. coli* colonies were selected by a colony picker and used to inoculate culture plates containing 150 μL of LB with 100 mg/L ampicillin per well. Cultures were grown overnight at 37 °C, and frozen stocks of these cultures were made by addition of 50 μL 60% (w/v) of glycerol to each well with a CCS Packard Automate.

2.4. Esterase Activity. *CLIPS-O esterase substrate synthesis.* C2-CLIPS-O (2-hydroxy-4-(*p*-nitrophenoxy)butyl-acetate), C3-CLIPS-O (2-hydroxy-4-*p*-nitrophenoxybutyl-propionate), C10-CLIPS-O (2-hydroxy-4-(*p*-nitrophenoxy)-butyldecanoate), and C16-CLIPS-O (2-hydroxy-4-(*p*-nitrophenoxy)butylpalmitate) substrates were synthesized from 4-bromobutene as described for the corresponding fluorescent umbelliferone derivatives.[14,15]

Assay Procedure. Substrates were prepared as 20 mM stock solutions in acetonitrile. All reagents and buffers were prepared in deionized milliQ water. Bovine serum albumin (BSA) was prepared as a stock solution (50 mg/mL) in water. $NaIO_4$ was freshly prepared as a 100 mM stock solution in water. Stock solutions of substrates (8 μL) were added to 74 μL of 200 mM PIPES buffer at pH 7.0. The reactions were initiated by adding 10 μL of samples. The reaction mixtures were incubated at 65 °C for 40 min. Samples were cooled on ice, and bovine serum albumin (BSA 2 mM), $NaIO_4$ (28 mM), and Na_2CO_3 (40 mM) were added to the mixture. After 10 minutes of incubation at room temperature, samples were centrifuged at 6000*g* for 5 min, and then transferred to a microplate. The OD of the yellow *p*-nitrophenol was recorded at $\lambda = 414$ nm using a Spectramax 190 microplate spectrophotometer (Molecular Devices).

2.5. Screening. 2.5.1. Microbial Screening. The presence and the localization of esterase activity were determined by removing 1 mL of cell suspension at various incubation times during culturing. Cells were centrifuged at 6000*g* for 10 min and resuspended in medium D to obtain a 10-fold concentration.

Concentrated cells or supernatant (10 μL) were assayed for esterase activity as described above.

2.5.2. Genomic DNA Library. Culture plates containing 150 μL of LB with 100 mg/L ampicillin per well were inoculated with 5 μL of the genomic DNA library frozen stock in *E. coli* MC1061. Cultures were grown for 12 h at 37 °C. Plates were centrifuged at 2360*g*, 4 °C, for 10 min. Cells were resuspended in 84 μL of 200 mM PIPES buffer (pH 7). A mix of C2- and C10-CLIPS-O substrates (8 μL) was added to each well, and plates were incubated at 65 °C for 40 min. The reaction was stopped by addition of a mix of 40 μL of 100 mM $NaIO_4$ and 40 μL of 200 mM Na_2CO_3

(6) Woese, C. R.; Kandler, O.; Wheelis, M. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 4576−4579.
(7) Sobek, H.; Görisch, H. *Biochem. J.* **1998**, *250*, 453−458.
(8) Manco, G.; Giosuè, E.; D'Auria, S.; Herman, P.; Carrea, G.; Rossi, M. *Arch. Biochem. Biophys.* **2000**, *373*, 182−192.
(9) Manco, G.; Adinolfi, E.; Pisani, F. M.; Ottolina, G.; Carrea, G.; Rossi, M. *Biochem. J.* **1998**, *332*, 203−212.
(10) Huddleston, S.; Yallop, C. A.; Charalambous, B. M. *Biochem. Biophys. Research Com.* **1995**, *216*, 495−500.
(11) Donaghy, J. A.; Bronnenmeier, K.; Soto-Kelly, P. F.; McKay, A. M. *J. Appl. Microbiol.* **2000**, *88*, 458−466.
(12) Maniatis, T.; Fristch, E. F.; Sambrook, J. *Molecular Cloning, A Laboratory Manual*; Cold Spring Harbor Laboratory: Cold Spring Harbor, New York, 1982.
(13) Hudson, J. A.; Morgan, H. W.; Daniel, R. M. *Syst. Appl. Microbiol.* **1989**, *11*, 250−256.
(14) Badalassi, F.; Wahler, D.; Klein, G.; Crotti, P.; Reymond, J.-L. *Angew. Chem.* **2000**, *112*, 4233−4236; *Angew. Chem., Int. Ed.* **2000**, *39*, 4067−4070.
(15) Wahler, D.; Badalassi, F.; Crotti, P.; Reymond, J.-L. *Angew. Chem.* **2001**, *113*, 4589−4592; *Angew. Chem., Int. Ed.* **2001**, *40*, 4457−4460.

containing 4.5 mg/mL BSA to each well. Plates were kept at room temperature for 15 min, and the $OD_{414\ nm}$ was determined using a Spectramax 190 microplate spectrophotometer (Molecular Devices).

3. Results and Discussion

3.1. Substrates. 3.1.1. Stability of Commercial Substrates. One of the most efficient methods to discover new biocatalysts consists of high-throughput screening of natural biodiversity. The majority of enzyme assays are based on chromogenic or fluorogenic substrates. Many fluorogenic or chromogenic enzyme substrates are commercially available and are generally highly reactive derivatives of phenols or anilines, such as butyric and oleic acid esters of 4-methylumbelliferone and 4-nitrophenol for lipases and esterases, and peptide-coumarinylamides and nitroanilides for proteases. One of the main drawbacks of these substrates is that their chemical reactivity makes them completely unstable under certain experimental conditions, such as extreme pH, high temperature, or presence of impurities in the extract medium. Screening for novel thermophilic enzymes cannot be achieved using such types of substrates because of the high level of background that is observed. Furthermore, due to this high level of background, screening for novel biocatalysts with these substrates may lead to the discovery of enzymes that are able to catalyze only the hydrolysis of the activated artificial substrates, and not substrates of lower reactivity as it is often the case with the substrates of interest (industrial substrates).

3.1.2. CLIPS-O Substrates. To overcome these limitations, we have designed a novel functional assay technology called CLIPS-O.[16] This is a versatile assay for enzymes that is based on a new type of chromogenic or fluorogenic enzyme-labile functional group which is chemically nonactivated. Thus, their spontaneous hydrolysis rate is very low in the absence of a specific catalyst. Moreover, their chemical structures are adapted to the ones of the targeted compounds. For instance, it is possible to identify enantioselective catalysts with the use of optically pure CLIPS-O substrates or to identify specific chain length esterases with the use of the appropriate CLIPS-O esterase substrates.

Such substrates are stable under a wide range of physicochemical conditions and thus minimise both false negative (high sensitivity) and false positive signals (no background due to high specificity and stability).

These functional assays are based on the enzymatic transformation of the substrate upon catalytic activity followed by a two-step process involving a periodate oxidation and a β-elimination reaction which allow the release of the chromogenic signal (Figure 1).

For our study, four types of esterase substrates were used: short- and long-chain ester substrates C2-CLIPS-O (2-hydroxy-4-(*p*-nitrophenoxy)butylacetate), C3-CLIPS-O (2-hydroxy-4-(*p*-nitrophenoxy)butylpropionate), C10-CLIPS-O (2-hydroxy-4-(*p*-nitrophenoxy)butyldecanoate) and C16-CLIPS-O

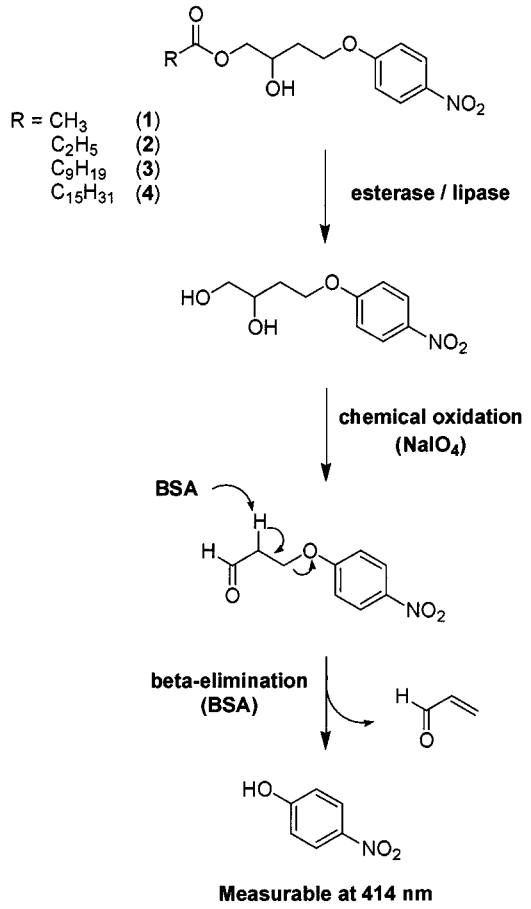

Figure 1. **Periodate-coupled chromogenic assay principle for esterase/lipase activity. C2-CLIPS-O (2-hydroxy-4-*(p*-nitrophenoxy*)*butylacetate (1)), C3-CLIPS-O (2-hydroxy-4-*(p*-nitrophenoxy*)*butylpropionate (2)), C10-CLIPS-O (2-hydroxy-4-*(p*-nitrophenoxy*)*butyldecanoate (3)) and C16-CLIPS-O 2-hydroxy-4-*(p*-nitrophenoxy*)*butylpalmitate (4)).**

(2-hydroxy-4-(*p*-nitrophenoxy)butylpalmitate) respectively (Figure 1). These ester substrates, with aliphatic acyl moieties and bearing no supplementary substituant on the butyl skeleton, were synthesized with an aim that they mimic ester substrates used in a specific industrial process. These substrates are hydrolyzed by specific esterases leading to the 1,2-diol derivative. This latter is then cleaved by oxidation with sodium periodate leading to the corresponding carbonyl derivative which is unstable and releases the colored product via a β-elimination reaction in the presence of BSA.

3.2. Screening on Whole Cells. Twenty strains of thermophilic microorganisms, belonging to various genera (including *Thermus, Bacillus, Thermoanaerobacter, Fervidobacterium*) were screened for esterase (or lipase) activity on C2-, C3-, C10-, and C16-CLIPS-O substrates. *Thermus* strain P1074 exhibited a high level of constitutive intracellular esterase activity (no esterase activity could be detected in the culture supernatant even in the presence of olive oil as an inducer). This thermophilic strain showed esterase activity on short- and long-chain CLIPS-O substrates (Figure 2). This could be due to the expression of a nonspecific enzyme, or to the expression of at least two kind of esterases (short-chain specific ones and long-chain specific ones). If so, the enzyme exhibiting activity on long-chain substrates

(16) Reymond, J. L.; Wahler, D.; Badalassi, F.; Nguyen, H. K. Method for releasing a product comprising chemical oxidation, method for detecting said product, and uses thereof. Patent WO 01/92563, 2001.

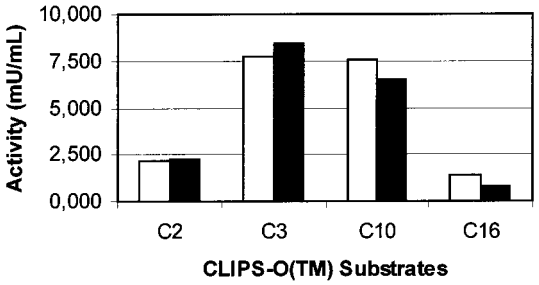

Figure 2. **Hydrolytic activity of esterase(s) produced by *Thermus* strain P1074 on various chain lengths of CLIPS-O substrates. (■) Measurement after 22 h of culture. (□) Measurement after 41 h of culture.**

may be a good candidate for industrial transformation of very long-chain (over C16) substrates at high temperature. To further characterize this thermophilic strain, we decided to create a genomic library and to screen for any gene coding for an esterase activity.

3.3. Screening of the Genomic Library. The genome size of *Thermus* strains ranges from 1.7 to 2.5 million bases.[17,18] Therefore we decided to screen a genomic DNA library of approximately 10 000 clones to ensure that at least one copy of the gene(s) of interest was found. A total of 9219 clones was screened for esterase activity using a mixture of C2- and C10-CLIPS-O substrates (easier to use in high-throughput screening than C16-CLIPS-O substrate). No background was observed during screening, which was not the case with commercial *p*-nitrophenyl derivatives. Three clones showed hydrolytic activity towards these two substrates. These clones were called Est-1, Est-2, and Est-3. The positive activity of these clones was confirmed by performing another activity test on an overnight grown culture with a mixture of C2- and C10-CLIPS-O substrates. A clone with no activity on both substrates was used as a negative control to estimate the level of background due to spontaneous substrate hydrolysis and potential endogenous esterase activity of *E. coli*.

The substrate specificity of each clone was then determined by performing tests using the C2- and C10-CLIPS-O substrates independently (Figure 3). A very low level of background was observed with the negative control. Est-1 was specific for the short-chain substrate (C2), with no significant activity on the long-chain one (C10). On the other hand, Est-2 and Est-3 exhibited significantly higher activity with long-chain substrates (high hydrolysis rates on C10-CLIPS-O) compared to the shorter-chain C2 substrate. This demonstrates that the CLIPS-O substrates enable the identification of strains producing esterases at high temperatures. Moreover, it enables the functional screening of a recombinant DNA library and the final biochemical characterization of the corresponding enzymes in terms of substrate specificity profile.

Long-chain (C10) enzymes show a good level of activity at temperatures above 65 °C which is consistent with

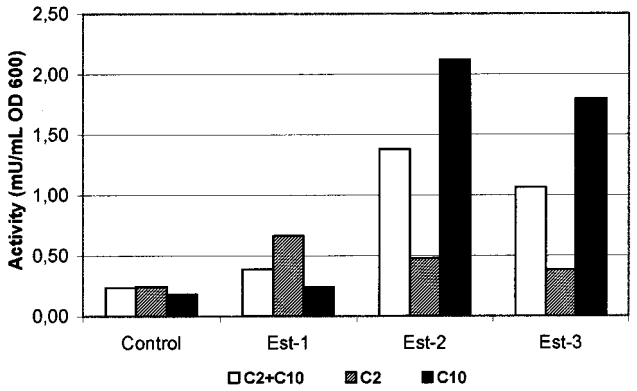

Figure 3. **Characterization of the substrate specificity of the three genomic clones of *Thermus* P1074 genomic library. The activities of the clones Est-1, Est-2, and Est-3 were determined on overnight cultures. The activity test was performed on a mixture of C2- and C10-CLIPS-O substrates. A clone with no activity on these substrates was used as negative control. The substrate specificity of each clone was determined performing the test with C2 and C10 substrates independently.**

the optimum growth temperature of the original strain, P1074.

Further enzyme characterization (such as thermostability, pH profile, solvent resistance, specific activity) can be carried out using the same assay technology.

4. Conclusions

"Nature has more imagination than our dreams." This means that screening of natural biodiversity is the leading way to discover novel and proprietary enzymes suited to industrial biocatalysis. The identification of natural extremophilic strains that produce interesting enzymes and the screening of the corresponding recombinant genomic libraries requires a robust functional screening tool. For such screening, the use of commercially available substrates is limited due to their high instability. To overcome this drawback, we have developed a novel substrate technology called CLIPS-O. This technology enables the use of designed highly specific molecules simulating the chemical structure and energetic state of industrially relevant substrates. Using this technology, we screened (within a few days) for novel esterases active at high temperatures which showed a high specificity on acyl carbon chain length. Very low to no background signal was observed during screening, and at least two novel esterases were identified. Final characterization of the biocatalyst active on long-chain substrate will demonstrate if they directly fit industrial requirements, especially in terms of thermostability. These enzymes may be engineered a second time to tightly adapt their properties to specific processes. This can be done using a directed evolution technology called L-Shuffling.[19] Directed evolution mimics natural evolution that occurs under selective pressure, but reduces the time scale from billions of years to weeks. Starting from original genes, this technology generates thousands of new recombinants that can be further screened

(17) Borges, K. M.; Bergquist, P. L. *J. Bacteriol.* **1993**, *175*, 103−110.
(18) Moreira, L.; Costa, M. S.; Sa-Correia, I. *Arch. Microbiol.* **1997**, *168*, 92−101.
(19) Dupret, D.; Masson, J. M.; Lefèvre, F. Method for obtaining in vitro recombined polynucleotide sequences, sequence banks, and resulting sequences. Patent WO 00/09679, 1998.

for their biochemical characteristics (acquired thermostability, solvent resistance, selectivity, etc.).

Combining original biodiversity, high-throughput screening techniques, and directed evolution to discover new and useful enzymes will certainly enable significant advances in industrial enzyme technology.

(CLIPS-O and L-Shuffling are trade marks of Proteus. CLIPS-O and L-Shuffling are patent pending technologies).

Acknowledgment

We thank Professor B. K. C. Patel for strain isolation, and Proteus' staff (S. Baffie, V. Cilia, V. Duvivier, M. Gilly, S. Pallas, C. Redares, J. Schmit) for their useful contribution to this work.

Received for review February 1, 2002.

OP020019H