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Screening and selection of a microbial lipase for the stereospecific hydrolysis of Verlukast

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SUMMARY

A search was implemented for a microbial lipase capable of bioconverting a diester (dimethyl 5-(3-(2-(7-chloroquinolin-2-yl)ethyl)phenyl)4,6-dithianonanedioate) to its S-ester acid, an intermediate in the production of Verlukast (a leukotriene receptor antagonist). Required properties of the sought-after enzyme included a high enantiomeric selectivity (e.e. >98%), the formation of only trace amounts of diacid and a high bioconversion rate. This search yielded 57 lipase-producing microorganisms, 18 of which presented detectable bioconversion activity. Thirteen of these microbes were selected for further study based upon their lipase production level and enzyme stability at harvest. Despite their common enzymatic property, namely the hydrolysis of triglycerides, these lipase preparations presented diverse ester acid specific synthesis rates (from <0.01 μg/unit/h to 0.98 μg/unit/h) and diacid formation levels (from 0% to 35%). One of these microbes, identified as *Pseudomonas aeruginosa* (strain MB 5001), was found to produce a lipase having all of the above-listed required properties. The initial fermentation process developed in shake flasks was rapidly and successfully scaled up in 23-liter laboratory bioreactors, achieving a maximum production of 35 units/ml of lipase after 48 h of cultivation.

INTRODUCTION

The production of extracellular lipase (triacylglycerol hydrolase, EC 2.1.1.3) is a widely distributed phenomenon among eukaryotic and prokaryotic microorganisms [2,13,18]. Historically, microbial lipases have mostly been employed by the food and detergent industry for purposes such as the ripening of cheese and additives to laundry detergent [22,27]. Only recently has their utilization by the chemical and pharmaceutical industry emerged [3,11]. Because of their specificity and enantioselectivity, lipases are now used in the preparation of asymmetric compounds whose large-scale synthesis would not be feasible and/or economical using conventional processes [3,5,11].

In an effort to obtain a highly enriched compound in the S-ester acid, Hughes et al. [8] reported the bioconversion of dimethyl 5-(3-(2-(7-chloroquinolin-2-yl)ethyl)phenyl)4,6-dithianonanedioate (diester) to the S-ester acid (Fig. 1) using several commercial lipases as catalysts. While the utilization of most of these commercially available lipases was impaired by an incomplete reaction, the production of large amounts of unwanted diacid, or the formation of R-ester acid above a permissible level, a small number of them did meet expectation. However, utilization of these enzymes in a large-scale process was economically unfavorable and prompted us to investigate the feasibility of producing an in-house lipase for this bioconversion.

The present communication describes the implementation and results of a search aimed at identifying a microorganism(s) capable of producing a lipase that can meet the strict requirements established for this specific conversion (i.e., high enantiomeric selectivity, low diacid formation, and high conversion yield). After examining 131 strains of bacteria, yeasts and fungi, *Pseudomonas aeruginosa* (strain MB 5001) was found to produce a lipase that met all of the above-mentioned requirements.

MATERIALS AND METHODS

Microorganisms

Microorganisms were obtained as lyophilized preparations from either our internal Culture Collection (Merck and Co., Rahway NJ), the American Type Culture Collection (Rockville, MD), the Northern Regional Research Laboratories (Peoria, IL), the International Mycological

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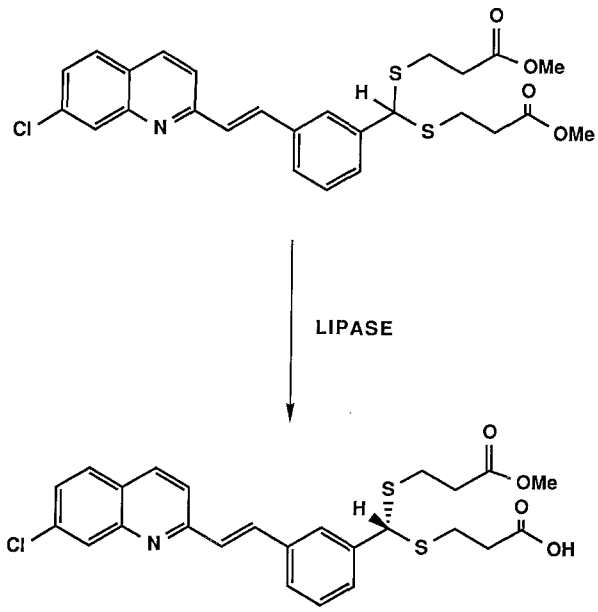

Fig. 1. Lipase catalyzed conversion of a diester (dimethyl 5-(3-(2-(7-chloroquinolin-2-yl)ethyl)phenyl)4,6-dithianonanedioate) to its S-ester acid.

Institute (Kew, Surrey, UK) or the Quartermaster Research and Development Center (Natick, MA) collections.

Prior to screening for lipase production, stock cultures prepared from reisolated colonies were obtained as follows: A 250-ml Erlenmeyer flask containing 50 ml of the appropriate seed medium (see below for composition) was inoculated with a lyophilized culture and incubated with shaking on an orbital shaker (180 rpm (New Brunswick Scientific, Edison, NJ)) until noticeable growth was achieved. Agar plates were subsequently streaked with a culture aliquot and incubated at the appropriate temperature until visible colonies developed. A single isolated colony was then used to inoculate a 250-ml Erlenmeyer flask as described above. After noticeable growth was achieved, a sterile glycerol solution (20%) was added to the culture (1:1, v/v) and cryogenic vials were filled with 1.2 ml of this mixture and stored at −70 °C. These frozen suspensions were routinely used as a source of inoculum only after culture purity was established.

Cultivation methods

Seed culture development. First-stage bacterial, yeast and fungal seed cultures were prepared by inoculating a 250-ml Erlenmeyer flask containing the appropriate growth medium (Nutrient broth (Difco, Detroit, MI), YM (Difco) and KF (17) media, respectively) with a 1-ml frozen cell suspension. These flasks were incubated aerobically (agitation of 220 rpm) at either 25 °C or 28 °C. The second-stage seed was prepared by transfering 10 ml of the 250-ml culture to a 2-liter flask containing 500 ml of appropriate growth medium. Aerobic cultivation (agitation of 180 rpm) was again performed at 25 °C or 28 °C for 48 to 72 h.

Lipase production. Lipase production flasks (2-liter Erlenmeyer flasks) containing 500 ml of the appropriate medium were inoculated with 10 ml of the second-stage seed culture and incubated with shaking at 180 rpm on an orbital shaker. Composition of the lipase production media varied according to the cultivated species. *Pseudomonas* species were cultivated at 25 °C in skim milk (100 g/l). Most yeast were cultivated at 25 °C using a lipase production medium composed of soybean meal (35 g/l), corn steep liquor (30 g/l), potassium phosphate (5 g/l) and ammonium sulfate (1 g/l). The yeast *Geotrichum* was cultivated in a medium made up of corn steep liquor (50 g/l), soybean oil (10 g/l) and ammonium nitrate (5 g/l) at 28 °C. Lipase production by *Aspergillus* and *Rhizopus* species was achieved at 25 °C and 22 °C, respectively, in Shipe's medium [23]. The addition of 20 ml/l of olive oil to the cultivation media enhanced lipase production of several yeasts and fungal species. Lipase production was evaluated daily on supernatant samples and the microorganisms were cultivated until lipase concentration reached a plateau.

Laboratory bioreactors. Lipase production by *Pseudomonas aeruginosa* was scaled up in 23-liter bioreactors (Chemap, South Plainfield, NJ). The production medium (15.5 l) consisted of 100 g/l skim milk (Difco) and 5 ml/l antifoam FD 62 (Hodag, Skokie, IL) and was inoculated with 500 ml of a second-stage seed. The reactor was operated at 20 °C, agitated at 350 rpm and aerated with 4 liters of air per min.

Analytical methods

Lipolytic activity. The assay mixture contained per liter: 740 ml of emulsified olive oil (Sigma, St Louis, MO), 75 ml of Trizma buffer (0.2 M, pH 7.6) (Sigma), and 185 ml of distilled water. Lipase activity was assayed in the culture supernatant obtained by centrifugation at 4000 rpm for 10 min at 4 °C (model RCB 5, Sorvall Instruments, Wilmington, DE). A volume of 1 ml of supernatant was added to a 250-ml flask containing 15 ml of assay mixture and incubated for 4 h at 37 °C. The reaction was stopped by the addition of 2.5 ml of ethanol, and the amount of fatty acids released was titrated with 0.05 M NaOH until a blue color developed (using Thymolphthlalein as an indicator). Background acidity was determined by performing the same titration on unincubated samples. One unit of lipase is defined as the amount of enzyme needed to release free fatty acids titrated with 1 ml of 0.05 M NaOH.

Bioconversion activity. The diester containing reaction

mixture was composed of (per liter): Triton X-100, 16 g; K_2HPO_4 (1 M, pH 7.6), 100 ml; water, 900 ml; and diester, 10 g. The solution was mixed at 25 °C for 30 min, filtered (medium fritted glass filter) and centrifuged for 10 min at 3500 rpm at 25 °C (Model TJ -6 Beckmann, Fullerton, CA). The supernatant was brought to the desired temperature and immediately used in the bioconversion assay. In order to prevent light-induced degradation of the diester, 250-ml amber flasks were routinely used to perform the assay. The reaction was initiated by the addition of an equal volume of prewarmed enzyme solution and substrate solution, and the flasks were incubated with agitation (220 rpm). The incubation temperature was identical to that used for the cultivation of the microorganisms. Samples (1 ml) were taken at regular time intervals, diluted in 9 ml of methanol, filtered (0.45 μm PUDF, Gelman, Ann Arbor, MI) and assayed by HPLC for diester and ester acid concentrations as previously described [8].

Protease activity. Supernatant proteolytic activity was assayed using the azocasein method as described by Ginther [6].

Enantiomeric excess. The enantiomeric excess was measured by HPLC as previously described by Hughes et al. [8].

RESULTS AND DISCUSSION

Lipase and bioconversion activities of screened microorganisms

One hundred and thirty-one strains of microorganisms were screened for lipase production (primary screen). Significant lipase activity (at least 2 units per ml) was detected in the supernatants of 43.5% of the culture broths (Table 1). Only a relatively low number of yeasts evaluated in this screen (27.2%) produced any significant amount of lipase, while higher percentages of lipase producers were found among the fungal and bacterial strains tested (51.2% and 93.8%, respectively). Comparable or higher percentages of lipase producers have been reported by several investigators [1,4,7,21]. Although the media employed in this screen supported the identification of lipase producers, more producing strains may have been detected if additional media had been employed [4,21].

Lipase containing cell-free fermentation broths were further evaluated for their ability to bioconvert the diester to its ester acid (secondary screen). These lipase preparations were subdivided into three major classes: presence or absence of bioconversion activity, amount of bioconversion activity, and level of diacid formation (undesirable side product). Bioconversion activity was detected in the fermentation broth supernatant of 31.5% of the strains evaluated (Table 1). Some preparations had lipase activity, but no bioconversion activity, presumably because the diester is not a substrate. Among the bioconversion active preparations, specific ester acid formation rates ranged from 0.01 μg/unit/h to 0.98 μg/unit/h (Table 3), suggesting a wide range of affinity for this synthetic substrate.

TABLE 1

Screen results: microorganisms presenting lipolytic and bioconversion activities

Microorganisms screened (bacteria, yeasts, fungi)	131
Lipase producers*	57
Lipase producers with detectable bioconversion activity**	18
Bacteria:	
Nocardia lactamdurans	
Pseudomonas aeruginosa (five strains)	
Pseudomonas fluorescens	
Fungi:	
Aspergillus niger	
Rhizopus oryzae	
Yeasts:	
Candida ernobi	
Candida humicola	
Candida rugosa	
Geotrichum candidum	
Yarrowia lipolytica (five strains)	

* Production of at least 2 units/ml of lipolytic activity.

** Measured by the detection of ester acid formation after 48 h of incubation.

During the bioconversion, several of the lipases produced only marginal amounts of diacid (perhaps because they did not recognize the ester acid as a substrate or because the hydrolysis rate was extremely slow). However, the lipase preparation from *Candida rugosa* produced large amounts of diacid (Table 2). When investigated in detail, the conversion kinetics of *C. rugosa* lipase showed that formation of diacid occurred after ester acid had already accumulated (Fig. 2), implying a step mechanism similar to triglyceride hydrolysis by pancreatic lipase [2]. All bioconverting yeast lipase preparations evaluated here produced diacid, indicating a higher degree of affinity toward the ester acid. This observation corroborates recent studies where yeast lipases were found to be positionally less specific than bacterial and fungal lipases [14].

Due to the large number and diversity of microbes investigated here, it should not be surprising to observe various rates and degrees of diester hydrolysis. Several investigators have reported differences in affinity and end-product formation patterns when evaluating commercial lipases in a specific conversion reaction [9,10,15,16,19,20,24,26]. Furthermore, it is worthwhile to underline that despite the relatedness of the two microorganisms, lipase preparations obtained from two Pseudomonas species (*P. aeruginosa* and *P. fluorescens*)

TABLE 2

Bioconversion rates of selected lipase preparations

Microorganisms	Ester acid formation rate (μg/ml/h)	Specific bioconversion activity (μg/unit of lipase/h)	Diacid formation (+++,++,+,−)*
P. aeruginosa (MB 5001)	15.2	0.98	−
P. fluorescens (MB 4721)	0.1	<0.01	−
A. niger (ATCC 1004)	2.7	0.50	−
G. candidum (IMI 96237)	2.5	0.08	+
R. oryzae (ATCC 20134)	0.5	0.04	+
C. ernobi (ATCC 20000)	0.6	<0.01	+
C. humicola (MY 1723)	1.7	0.20	+
C. rugosa (ATCC 14830)	3.6	0.70	+++
Y. lipolytica (ATCC 60595)	1.0	0.03	+

* Amount of diacid formed: +++: >25 μg/ml; ++: 8–25 μg/ml; +: 1–8 μg/ml; −: <1 μg/ml.

exhibited remarkably different affinity for the diester, as indicated by their respective ester acid specific formation rates (Table 2).

Finally, lipase preparations obtained from five strains of *Pseudomonas aeruginosa* were found to catalyze the formation of ester acid with the highest specific conversion rate of all preparations tested (by at least 4-fold) as well as to produce only trace amounts of diacid (Table 2). Consequently, these lipase preparations were further evaluated for their stereospecificity in the conversion of the diester to the ester acid in a tertiary screen. All were found to produce the desired S-ester acid in highly pure form (e.e. >98%) (Table 3).

Strain selection and fermentation scale-up

All five *P. aeruginosa* strains were evaluated for their lipase and protease production levels. Lipase degradation in fermentation broth by proteases is a reported phenomenon [12] and, the use of a high-lipase/low-protease producer can positively impact downstream processing by enhancing enzyme recovery yields. Three of the five *P. aeruginosa* strains tested met these two requirements (Table 4). Strain MB 5001 was finally selected as the lipase producer over the other two based solely on proprietary considerations.

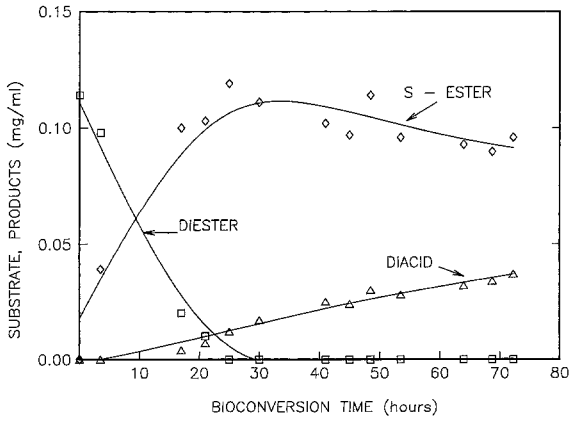

Fig. 2. Time-course of (dimethyl 5-(3-(2-(7-chloroquinolin-2-yl)ethyl)phenyl)4,6-dithianonanedioate) conversion to its ester acid and diacid by *Candida rugosa* lipase preparation.

TABLE 3

Enantiomeric excess determinations

P. aeruginosa (strain No.)	S-ester (%)	R-ester (%)	Enentiomeric excess* (%)
MB 4698	99.3	0.7	98.6
MB 4821	99.6	0.4	99.2
MB 4822	99.6	0.4	99.2
MB 5000	99.5	0.5	99.0
MB 501	99.6	0.4	99.2

* Enantiometric excess (%) = [S-ester (%) − R-ester (%)].

TABLE 4

Pseudomonas aeruginosa lipolytic and proteolytic activities

P. aeruginosa (strain No.)	Lipase production* (units/ml)	Protease production* (units/ml)
MB 4698	21.5	17.3
MB 4821	16.5	1.2
MB 4822	18.0	2.1
MB 5000	16.0	5.1
MB 5001	18.5	0.9

* Lipolytic and proteolytic activities were measured on supernatants after 50.5 and 67.5 h of cultivation, respectively.

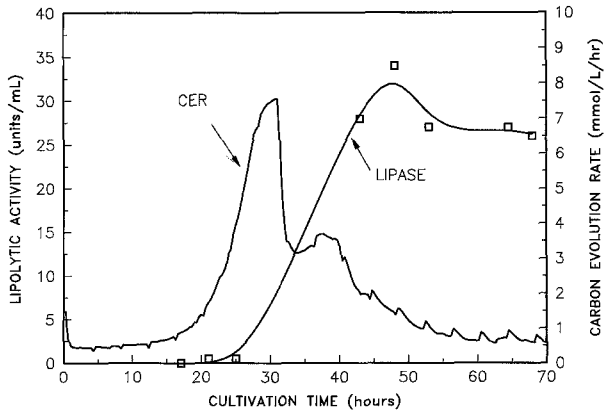

Fig. 3. Lipase production time-course by *Pseudomonas aeruginosa* cultivated on skim milk in a 23-liter bioreactor.

The initial shake-flask production process was scaled up in laboratory fermentors (23-liter) employing skim milk as production medium. Increased lipase production was obtained in bioreactors when compared with shake-flask performances (35 units/ml vs. 18.5 units/ml). Lipase formation occurred between 25 h and 45 h following inoculation with most of it synthesized during late growth phase as measured by carbon evolution rate (Fig. 3). It is worthwhile to notice that most of the lipase produced was remarkably stable under prolonged incubation, a fact that should be credited to the low proteolytic activity of the selected strain (Table 4).

In conclusion, after an extensive screen, the bacterium *Pseudomonas aeruginosa* MB 5001 was found to produce a lipase capable of bioconverting dimethyl 5-(3-(2-(7-chloroquinolin-2-yl)-ethyl)phenyl)4,6-dithianonanedioate (diester) to its S-ester acid with the formation of only trace amounts of undesirable diacid. The bioconversion kinetics of that lipase preparation were superior to all those of other microbial species tested in this study. This novel approach in identifying a lipase specifically suited for an enantio-conversion underlines the benefits of screening microorganisms when this activity either cannot easily be found among commercial lipases, or when the utilization of a commercial enzyme may be too costly.

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