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Optimization of lipase production by *Pseudomonas aeruginosa* MB 5001 in batch cultivation

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SUMMARY

The production of *Pseudomonas aeruginosa* MB 5001 extracellular lipase was optimized by batch cultivation employing shake flasks and 23-L bioreactors. This enzyme efficiently and selectively bioconverts dimethyl 5-(3-(2-(7-chloroquinolin-2-yl)ethyl)phenyl)4,6-dithianonanedioate (diester) to its (S)-ester acid. Process development studies focused on the identification and optimization of the physicochemical parameters required to achieve maximum lipase production. Of the media evaluated, a peptonized milk-based medium was found to support excellent lipase production and stability. Medium composition and process parameters that supported optimal lipase production were different from those supporting maximum biomass formation. Of the parameters investigated, dissolved oxygen tension had the most significant and unexpected impact on lipase production. Elevated lipase production was achieved when *P. aeruginosa* MB 5001 was cultivated in a dissolved oxygen limited environment. Overall, these process development studies resulted in a 100% increase in lipase production when compared to the original shake flask process employing skim milk.

INTRODUCTION

Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) which hydrolyze long chain triglycerides are produced by a wide variety of microorganisms [17]. Traditionally, these microbial lipases have been used in the food and detergent industry for the ripening of cheese [18,24] and as laundry detergent additives [25]. Recently, these enzymes have become of interest to the chemical and pharmaceutical industries because of their ability to hydrolyze ester bonds, trans-esterify triglycerides, resolve racemic mixtures, and synthesize ester and peptide bonds [12,18].

Recently, our laboratory identified a strain of *Pseudomonas aeruginosa* (MB 5001) which produces a specific extracellular lipase which converts dimethyl 5-(3-(2-(7-chloroquinolin-2-yl)ethyl)-phenyl)4,6-dithianonanedioate (diester) to its ester acid, an intermediate in the synthesis of Verlukast, a leukotriene receptor antagonist [5]. Selection of the lipase producing microorganism and characterization of the enzyme have been previously reported [5,13]. This communication describes the optimization of this lipase-producing process employing the nontraditional cultivation of the microorganisms under limiting dissolved oxygen tension.

MATERIALS AND METHODS

Chemicals

The complex medium ingredients used were: nutrient broth (NB), dehydrated skim milk, yeast extract, beef extract (Difco, Detroit, MI, USA), and peptonized milk (Sheffield Products, Norwich, NY, USA). Sigma lipase substrate, Trizma buffer, and thymolphthalein indicator solution were obtained from Sigma Diagnostics (St Louis, MO, USA). All other chemicals were of reagent grade and were purchased from Fisher Scientific (Springfield, NJ, USA).

The antifoams used were: FD 62 (Hodag, Skokie, IL, USA); SAG 471, SAG 4130, and SAG 5693 (Union Carbide, Danbury, CT, USA); Chemax DF-10 and Chemax DF-30 (Chemax Inc., Greenville, SC, USA); P2000 (Dow Chemical, Midland, MI, USA); Mazu 210S (Mazur Chemicals Inc., Gurnee, IL, USA); Cod-liver oil and Soybean oil (Sigma Chemical Co., St Louis, MO, USA); Mazola corn oil (CPC International Inc., Englewood Cliffs, NJ, USA); Bertolli olive oil (Lucca, Italy); Lard burning oil (PFAU, Jeffersonville, IN, USA). Diester [dimethyl 5-(3-(2-(7-chloroquinolin-2-yl)-ethyl)phenyl)4,6-dithianonanedioate] was obtained from Merck and Co. (Rahway, NJ, USA).

Cultivation media

Seed medium. Inocula were developed in NB medium (8 g L^{-1}). The medium was dispensed into 250-ml or 2-L Erlenmeyer flasks (50 ml and 500 ml, respectively), capped with cotton plugs and sterilized by autoclaving at 121 °C for 20 and 25 min respectively.

Production media

Several production media were evaluated for their ability to support lipase production. Medium PM contained 80 g of peptonized milk per liter of distilled water. Medium SM contained 100 g of dehydrated skim milk per liter of distilled

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water. Medium BYE contained (per liter of distilled water): peptone, 50 g; yeast extract, 5 g; beef extract, 3 g; $MgSO_4 \cdot 7H_2O$, 1 g; K_2HPO_4, 2 g; and $CaCl_2$, 0.1 g. The pH of the medium was adjusted to 7.2. Medium M9-MOD contained (per liter of distilled water): Na_2HPO_4, 7 g; KH_2PO_4, 3 g; NH_4Cl, 1 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $CaCl_2$, 0.02 g; $NaCl_2$, 0.5 g; 3-(*N*-Morpholino propanesulfonic acid) [MOPS], 5 g; and glucose, 4 g. Glucose was sterilized separately from the remaining ingredients. The pH of the medium was adjusted to 7.0 prior to autoclaving. All production media were dispensed (500 ml) into 2-L Erlenmeyer flasks capped with cotton plugs, and sterilized by autoclaving at 121 °C for 25 min.

Inoculum development

A frozen cell suspension of *P. aeruginosa* MB 5001 was thawed at room temperature and added to a 250-ml unbaffled Erlenmeyer flask containing 50 ml of NB medium. The culture was incubated aerobically for 24 h at 28 °C on a rotary shaker at 220 rpm (2-inch throw). A second stage seed was prepared by transferring 25 ml of the 24-h culture to a 2-L Erlenmeyer flask containing 500 ml of NB medium and incubating it aerobically for 24 h at 28 °C on a rotary shaker at 180 rpm (2-inch throw). The final seed stage was prepared similarly to the second stage seed but was incubated for only 16 h. The production medium (500 ml) was inoculated with 25 ml of a 16-hour-old third stage seed. The cultures were incubated aerobically at 25 °C on a rotary shaker at 180 rpm (2-inch throw).

Laboratory fermentations

A 23-L fermentor (Chemap Inc., South Plainfield, NJ, USA) was filled with 15 L of PM medium and 30 ml of SAG 471 antifoam, and sterilized in situ at 123 °C for 25 min. The reactor was inoculated with 500 ml of a third stage seed. Standard operating conditions of the fermentor were: 25 °C; 4 L air min^{-1}; agitation 500 rpm. Dissolved oxygen tension was monitored using a polarographic probe (Ingold Electronics, Wilmington, MA, USA) and was expressed as a percentage of its initial value (100%). Dissolved oxygen tension was maintained to its desired value throughout the fermentation cycle by automatic computer control. Samples to evaluate biomass, lipase and bioconversion activities were acquired using an automatic whole broth multi-fermentor sampling system as previously described [23].

Analytical methods

Biomass was estimated by optical density at 660 nm using a Hewlett Packard 8451A Diode array spectrophotometer (Hewlett Packard, Cupertino, CA, USA).

The procedure used to estimate the amount of lipase produced was based on the method of Tietz and Fiereck [28]. A whole broth sample was centrifuged for 5 min at maximum speed in a Brinkman Eppendorf centrifuge, model 5415 (Brinkman Instruments, Inc., Westbury, NY, USA). A volume of 1 ml of the supernatant was added to a 250-ml flask containing: water, 2.5 ml; Sigma lipase substrate, 10 ml; Trizma Buffer, 1.0 ml. The flask was capped with aluminum foil and incubated at 37 °C on a rotary shaker at 180 rpm (2-inch throw) for 4 h. Immediately following incubation, a volume of 3 ml of 95% ethanol and four drops of thymolphtalein indicator solution were added to the flask and the contents were mixed. The quantity of fatty acids liberated was determined by titration with 0.05 N sodium hydroxide until a slight but definite blue color was obtained. One unit of lipase was defined as 1 ml of 0.05 N sodium hydroxide added.

Bioconversion activity was measured as follows. The substrate mixture containing (per liter): Triton X-100, 16 g; K_2HPO_4 (1 M, pH 7.6), 100 ml; distilled water, 900 ml; and diester, 10 g; was mixed at 25 °C for 30 min, filtered (medium porosity glass filter) and centrifuged for 10 min at 3500 rpm (Model TJ-6 Beckmann Fullerton, CA, USA). The supernatant was brought to the desired temperature (37 °C) and immediately used. The assay was performed in 250 ml amber flasks at 37 °C with agitation. The bioconversion was initiated by the addition of 10 ml of test enzyme (prewarmed at 37 °C) to 10 ml of substrate solution. Samples (1 ml) were taken at regular time intervals, diluted in 9 ml of methanol, filtered and assayed by HPLC for diester and ester acid concentrations as previously described [12]. A unit of bioconversion is defined as the amount of enzyme catalyzing the formation of 0.01 mg ester acid h^{-1}.

RESULTS

Medium and antifoam screen

Initial process development studies focused on replacing skim milk (SM) production medium with a less expensive substitute that was amenable to downstream processing (i.e. avoidance of membrane fouling [10]). Several production media containing alternate nutrient sources were investigated at the 2-L shake flask scale (Table 1). A 1.4-fold increase in lipolytic activity was achieved when employing both PM and BYE media (Table 1). Even though both media supported high lipase production, desired enzyme stability (extended stability after peak production) was only achieved in PM medium (Fig. 1).

Prior to scale-up in stirred bioreactors, a screen for

TABLE 1

Effect of production medium composition on lipase production by *P. aeruginosa* MB 5001 (2-L flask scale)*

Production medium**	Maximum lipolytic activity (units ml^{-1})
PM	26.0
BYE	26.0
SM	18.5
M9-MOD	6.5

*Values presented are an average of two flasks.
**Media compositions and lipase activity units are described in the Materials and Methods section.

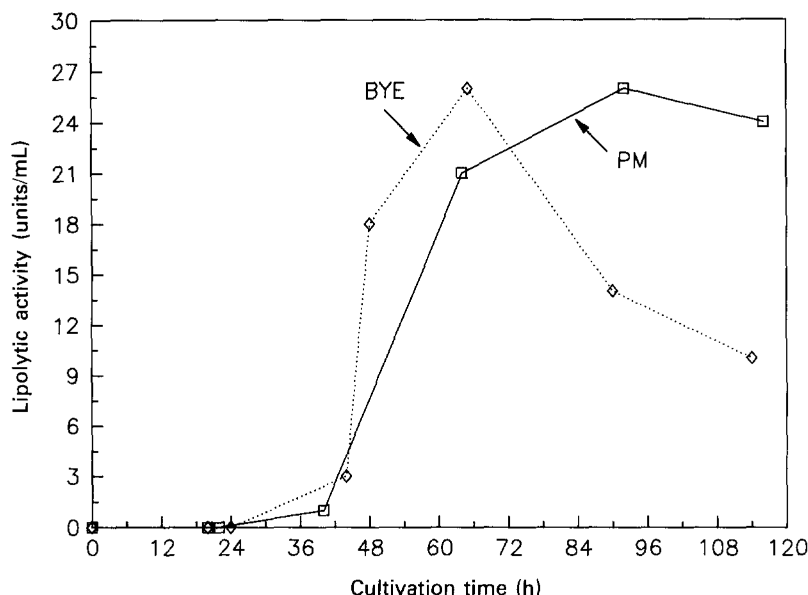

Fig. 1. Lipase production kinetics by *P. aeruginosa* MB 5001 cultivated in PM and BYE media (2-L shake flask scale).

antifoam was conducted in 2-L shake flasks employing PM medium. Three basic types of antifoams were screened: silicones, polyglycols, and natural oils. The majority of the silicone and polyglycol antifoams were found to have little or no effect on lipase production, while all of the natural oils were found to be inhibitory to lipase production (Table 2). While the routinely used P2000 supported acceptable lipase production (Table 2), it caused membrane fouling in downstream processing and was not selected. The silicone-based antifoam SAG 471 supported good lipase activity (Table 2), did not interfere with downstream processing, and was selected for the remainder of these process development studies.

TABLE 2

Effect of antifoam agents on lipase production by *P. aeruginosa* MB 5001*

Antifoam	Concentration ($ml\ L^{-1}$)	Lipolytic activity (% control)
None	0	100
FD 62	5	100
SAG 471	5	127
SAG 4130	5	94
P2000	5	102
SAG 5693	5	100
Mazu 210 s	5	94
Chemax DF-10	5	92
Chemax DF-30	5	102
Cod liver oil	1	89
Corn oil	1	22
Olive oil	1	89
Soybean oil	1	80
Lard oil	1	58

*Experiments were conducted in 2-L shake flasks containing 80 g L^{-1} of peptonized milk incubated at 25 °C. Maximum lipase titers are presented.

Scale-up and process optimization

Initial scale-up studies. Initial scale-up of the shake flask fermentation process was successfully achieved in 23-L bioreactors, employing PM production medium and the antifoam SAG 471. A maximum optical density of 9.5 was achieved 24 h after inoculation (Fig. 2A). Respiratory activity peaked at 21 h with an oxygen uptake rate (OUR) reaching a maximum of 105 mmol L^{-1} h^{-1} (Fig. 2A). Lipase production occurred in the late exponential growth and early stationary phases (Fig. 2A,B). Supernatant lipolytic and bioconversion activities reached a maximum of 23 units ml^{-1} and 16 units ml^{-1} respectively, approximately 30 h after inoculation (Fig. 2B).

Process sensitivity to dissolved oxygen. The effect of dissolved oxygen tension on lipase production (as measured by lipolytic and bioconversion activities) was investigated in bioreactors under three conditions: unlimited, temporarily limited, and limited. In an oxygen-unlimited fermentation, the dissolved oxygen tension was maintained above 25% throughout the fermentation cycle (Fig. 3A). Under these conditions, a maximum optical density of 10.6 was obtained, and supernatant lipolytic and bioconversion activities reached a maximum of 25.5 units ml^{-1} and 0.07 units ml^{-1}, respectively (Table 3). In an oxygen-limited fermentation, the dissolved oxygen tension was allowed to decrease to 0% and was maintained at that value for the remainder of the growth phase (11 h) (Fig. 3B). Under these conditions, a maximum optical density of 9.3 was achieved, and supernatant lipolytic and bioconversion activities reached a maximum of 37 units ml^{-1} and 0.15 units ml^{-1}, respectively (Table 3). In a temporarily oxygen-limited fermentation, the dissolved oxy-

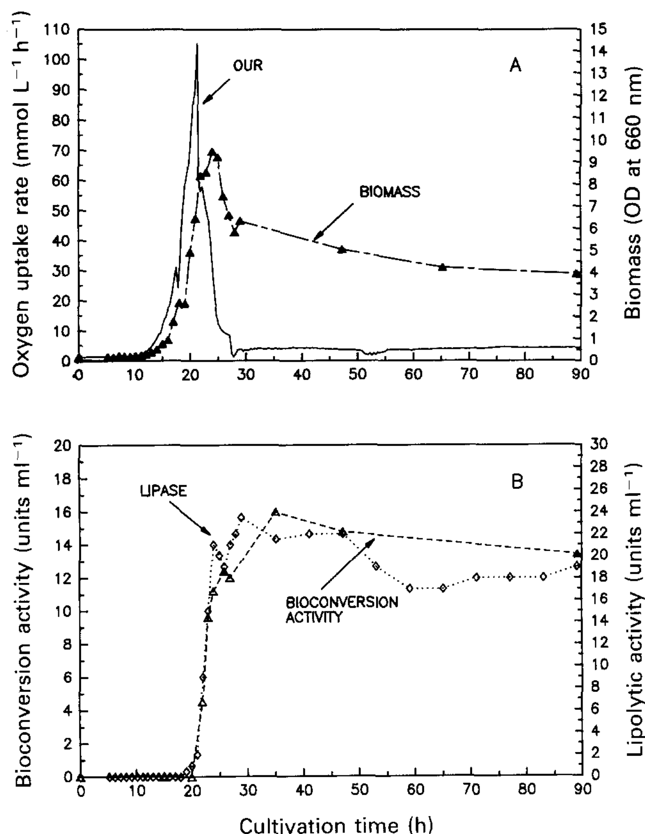

Fig. 2. A,B. Growth and lipase production by *P. aeruginosa* MB 5001 cultivated in PM medium (23-L bioreactor scale).

gen tension was allowed to decrease to 0% and was then immediately increased above 0% by ramping up the agitation (Fig. 3C). Under these conditions, a maximum optical density of 9.0 was achieved, and supernatant lipolytic and bioconversion activities reached a maximum of 23.5 units ml^{-1} and 0.07 units ml^{-1}, respectively (Table 3).

These data indicate that higher lipolytic and bioconversion activities were achieved when *P. aeruginosa* MB 5001 was cultivated in a dissolved oxygen-limited environment (Table 3). Because biomass production also was affected by oxygen availability, a 1.7-fold increase in specific activity was achieved in the oxygen-limited fermentation (Table 3).

Process sensitivity to pH, temperature and medium strength. These three parameters were investigated using the optimal oxygenation conditions previously defined. Cultivation pH affected both maximum lipase production and cellular growth. Maximum biomass production and growth rate were achieved when pH was maintained at 7.5, while maximum lipolytic activity was achieved when pH was maintained at 7.0 (Table 4). Cultivation temperature also affected both biomass and lipase production. Higher biomass production and specific growth rate were achieved at 30 °C, while maximum lipolytic and specific activities were achieved at 25 °C (Table 5).

Greater volumetric biomass production was achieved when the concentration of peptonized milk was increased from 80 g L^{-1} to 240 g L^{-1}, while lower concentrations of peptonized milk (40 g L^{-1} and 60 g L^{-1}) supported higher specific biomass yields (OD g peptonized milk^{-1}), specific activities, and lipase yields (Table 6). Optimal lipase volumetric production was achieved when employing 80 g L^{-1} of peptonized milk.

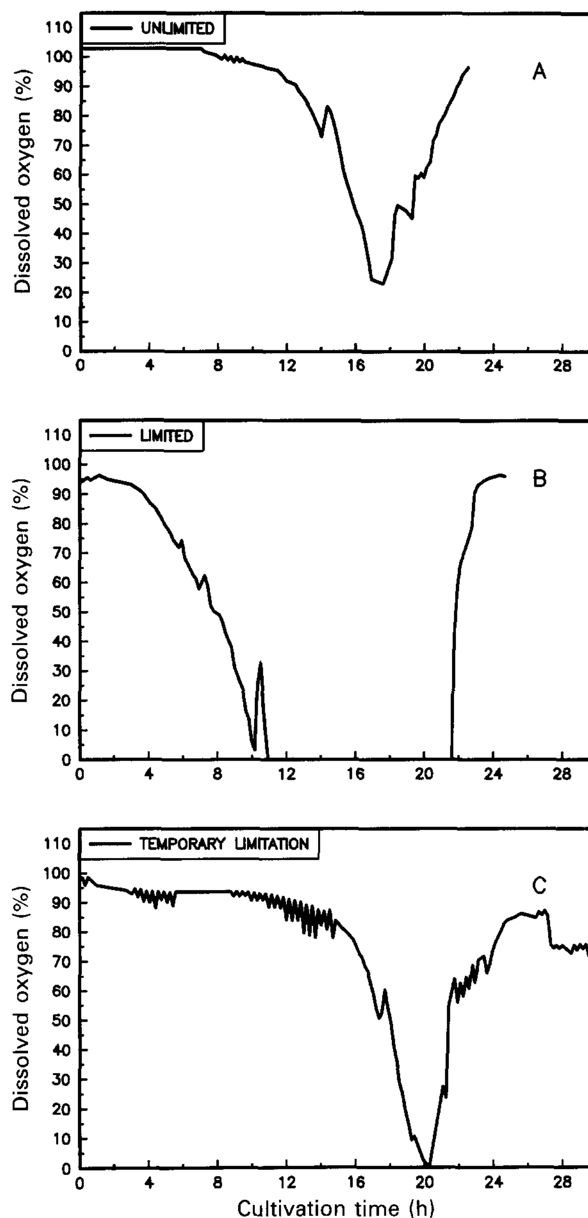

Fig. 3. Dissolved oxygen tension profiles (23-L bioreactor scale). A, Unlimited dissolved oxygen. Initial operating conditions were: agitation, 500 rpm and aeration, 4 L min^{-1}. The dissolved oxygen tension was maintained above 25% by computer controlled ramping of the agitation up to 700 rpm and by manual increase of the aeration up to 10 L min^{-1}. B, Limited dissolved oxygen. Initial operating conditions were: agitation, 300 rpm and aeration, 4 L min^{-1}. Agitation was increased to 500 rpm 24 h after inoculation. C, Temporarily limited dissolved oxygen. Initial operating conditions were: aeration, 4 L min^{-1} and agitation, 325 rpm. After dissolved oxygen tension reached 0% (20 h), the agitation was immediately increased to 550 rpm and maintained at that value for the rest of the fermentation cycle.

TABLE 3

Effect of dissolved oxygen on lipase production by *P. aeruginosa* MB 5001*

Dissolved oxygen tension	Lipolytic activity (units ml^{-1})	Bio-conversion activity (units ml^{-1})	Specific activity (lipolytic activity unit OD^{-1})
Unlimited	25.5	0.07	2.3
Temporary limitation	23.5	0.07	2.6
Total limitation	37.0	0.15	4.0

*Experiments were conducted in 23-L fermentors. The values presented are an average of two fermentations.

TABLE 4

Effect of pH on lipase production by *P. aeruginosa* MB 5001*

pH value	Lipolytic activity (units ml^{-1})	Optical density (660 nm)	Doubling time (h)
6.0	29.0	7.0	2.0
6.5	30.5	7.8	1.6
7.0	37.0	7.9	1.6
7.5	29.0	8.7	1.5
8.0	22.5	6.0	2.0

*Experiments were conducted in 23-L fermentors. The values presented are an average of two fermentations.

TABLE 5

Effect of temperature on lipase production by *P. aeruginosa* MB 5001*

Temperature (°C)	Maximum OD (OD 660 nm)	Lipolytic activity (units ml^{-1})	Specific activity (units OD^{-1})	Specific growth rate (h^{-1})
20	5.60	18	3.2	0.28
25	8.10	31	3.8	0.39
30	9.03	22	2.4	0.46
35	6.40	15	2.3	0.31
40	6.43	8	1.3	0.35

*Experiments were conducted in 23-L fermentors. The values presented are an average of two fermentations.

DISCUSSION

Lipase production by *P. aeruginosa* MB 5001 was enhanced by optimization of the chemical (medium) as well as the physical (pH, temperature, dissolved oxygen tension) parameters of the fermentation process. When implemented in 23-L bioreactors, these optimized process parameters resulted in a 100% increase in lipase production over the original skim milk based medium.

Lipase production and stability were found to be sensitive to medium composition. This study indicates that complex production media supported higher lipase production and stability, corroborating previous studies [1,6,8,11,16,20,21, 27,30]. The increased stability of the enzyme observed in peptonized milk may be attributed to formation of stabilizing association complexes between lipase and medium components, such as polysaccharides and divalent cations [2,4]. Supplementing the production medium with natural oils, which can act as lipase inducer [3,9,14,19,22] and as antifoam agent [29], resulted in reduced lipase production, an inhibition possibly caused by the release of free fatty acids [7,9].

As expected, lipase production by *P. aeruginosa* MB 5001 was influenced by several process parameters (dissolved oxygen, pH, and temperature). Interestingly, the conditions that supported high lipase production were different from those supporting high biomass production. Of the parameters investigated, dissolved oxygen had an unexpected but significant impact on lipase production by *P. aeruginosa*. While other microorganisms and strains of *Pseudomonas* require elevated oxygen tension to achieve maximum lipase production [9,14,26,27], optimum lipase production was achieved when *P. aeruginosa* MB 5001 was cultivated under limiting dissolved oxygen tension.

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TABLE 6

Effect of peptonized milk (PM) concentration on lipase production by *P. aeruginosa* MB 5001*

PM concentration ($g\ L^{-1}$)	Lipolytic activity (units ml^{-1})	Optical density (660 nm)	Specific activity (lipase OD^{-1})	Lipase yield (units g PM^{-1})	Cell yield (OD g PM^{-1})
40	20.0	5.2	3.8	0.50	0.13
60	26.5	5.7	4.6	0.44	0.10
80	32.0	7.5	4.3	0.40	0.09
160	31.0	12.6	2.4	0.19	0.08
240	22.0	15.2	1.4	0.09	0.06

*Experiments were conducted in 23-L fermentors. The values are an average of two fermentations.

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