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Stereoselective enzymatic esterification of 3-benzoylthio-2-methylpropanoic acid

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Summary. A key intermediate, *S*-(−)-3-benzoylthio-2-methylpropanoic acid (**1**) was made in high optical purity by the lipase-catalyzed stereoselective esterification of racemic **1** with methanol in an organic solvent system. Among various lipases evaluated, Amano P-30 lipase from *Pseudomonas* sp. efficiently catalyzed the esterification of **1** to yield *R*-(+) methyl ester and unreacted *S*-(−) **1**. A reaction yield of 40 mol% and an optical purity of 97.2% were obtained for compound **1** at a substrate concentration of 0.1 M (22 mg/ml). Lipase P-30 was immobilized on Accurel polypropylene (PP) and the immobilized enzyme was reused (23 cycles) in the esterification reaction without loss of enzyme activity, productivity or optical purity. Among various solvents evaluated, toluene was found to be the most suitable organic solvent and methanol was the best alcohol for the esterification of racemic **1** by immobilized lipase. Substrate concentrations as high as 1.0 M were used in the esterification reaction. When the temperature was increased from 28° C to 60° C, the reaction time required for the esterification of 0.1 M substrate decreased from 16 h to 2 h. On increasing the methanol to substrate molar ratio from 1:1 to 4:1, the rate of esterification decreased. A lipase fermentation using *Pseudomonas* sp. ATCC 21808 was developed. In the batch-fermentation process, 56 units/ml of extracellular lipase activity was obtained. A fed-batch process using soybean oil gave a significant increase in the lipase activity (126 units/ml). Crude lipase recovered from the filtrate by ethanol precipitation and immobilized on Accurel PP was also effective: *S*-(−) compound **1** was obtained in 35 mol% yield and 95% optical purity.

Introduction

Captopril is designated chemically as (2S)-1-[3-mercapto-2-methylpropionyl]-L-roline (**2**). It is used as an antihypertensive agent through suppression of the renin-angiotensin-aldosterone system (Ondetti and Cushman 1981; Ondetti et al. 1977). Captopril prevents the conversion of angiotensin I to angiotensin II by inhibition of angiotensin converting enzyme (ACE) (Cushman and Ondetti 1980). The potency of captopril **2** as an inhibitor of ACE depends critically on the configuration of the mercaptoalkanoyl moiety; the compound with the *S*-configuration is about 100 times more active than its corresponding *R*-enantiomer (Cushman et al. 1977).

The required (2*S*)-3-mercapto-2-methylpropionic acid moiety has been prepared from the microbially-derived chiral (2*R*)-3-hydroxy-2-methylpropionic acid, which is obtained by the hydroxylation of isobutyric acid (Goodhue and Schaeffer 1971; Schimazaki et al. 1982; Hasegawa et al. 1982).

The use of extracellular lipases of microbial origin to catalyze the enantioselective hydrolysis of 3-acylthio-2-methylpropanoic acid ester in an aqueous system has been demonstrated to produce optically active 3-acylthio-2-methyl propanoic acid (Sih 1987; Gu et al. 1986; Sakimas et al. 1986).

In this report, we describe the stereoselective esterification of racemic 3-benzoylthio-2-methylpropanoic acid (**1**) in an organic solvent system to yield *R*-(+) methyl ester and unreacted acid enriched in the desired *S*-(−) enantiomer. *S*-(−) 3-Benzoylthio-2-methylpropanoic acid is a key intermediate for the synthesis of **2** (Moniot 1988) or zofenopril (**3**) (Ondetti et al. 1982); both are antihypertensive drugs (Fig. 1).

Materials and methods

Enzyme source. Crude lipases AK, P-30, CES-30 and CE from *Pseudomonas* sp., lipase N from *Rhizopus* sp., lipase MAP from *Mucor* sp., lipase AY from *Candida* sp., lipase CE from *Humicola langinosa*, lipase G from *Penicillium* sp., and lipase APF-12 from *Aspergillus niger* were obtained from the Amano International Enzyme Co., Troy, Va., USA. Lipase B, from *Pseudomonas fluorescens* and lipase F9 from *Aspergillus* sp. were obtained from Enzymatics, Cambridge, UK.

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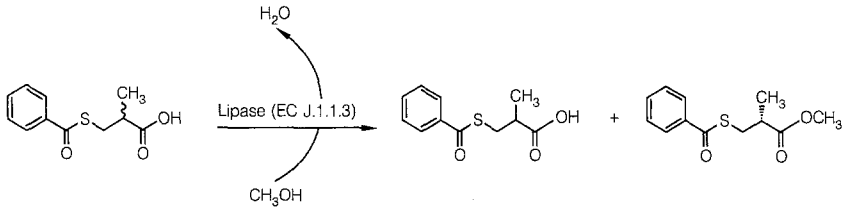


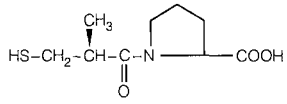
Racemic 3-benzoylthio-2-methylpropanoic acid

(Racemic **1**)

S-(-)-3-benzylthio-2-methylpropanoic acid

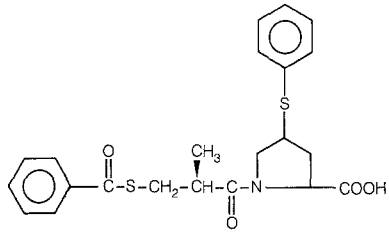
(S-(-) **1**)

R-(+)-3-benzoylthio-2-methylpropanoic acid methyl ester



(2**S**)-[1-[3-mercapto-2methyl-1-oxopropyl]-L-proline

2 Captopril



(1**R**)-[1,2α,4α]-1-[3-(benzoylthio)-2-methyl-1-oxopropyl]-4-(phenylthio)-L-proline

3 Zofenopril

Fig. 1. Structure of captopril (**2**) and zofenopril (**3**)

Lipase activity assay. The lipase activity was determined by titrimetric assay as recommended by Sigma (St. Louis, Mo., USA). Using a suitable pH-stat (Metrohm pH-stat, Brinkmann Instrument Co., Westbury, New York), and magnetic stirrer, 15 ml reagent A (50 ml distilled water, 50 ml Sigma lipase substrate, 20 ml of 3 M NaCl, 20 ml of 1.5% (w/v) sodium taurocholate, and 20-ml of 0.075 M $CaCl_2 \cdot 2H_2O$) adjusted to pH 8.0 at 37° C were pipetted into a titration vessel thermostated at 37° C. A suitable amount of enzyme was added and the timer was started when the pH of the reaction mixture dropped to 7.7. The time (t) required for the consumption of 500 µl of 0.1 M NaOH while maintaining the pH at 7.7 (37° C) was recorded. Enzyme activity in units/mg was defined as:

$$\text{units/mg} = \frac{0.1 \times 500}{\text{mg of lipase added} \times t\ (\text{min})}$$

One unit under the above conditions will hydrolyze 1.0 microequivalent of fatty acids from a triglyceride in 1 h at pH 7.7 and 37° C.

Immobilization of lipase. Three different resins, XAD-7 (Amberlite non-ionic polymeric adsorbent, 20–60 mesh, polyacrylate), XAD-2 (Amberlite non-ionic polymeric adsorbent, 20–60 mesh, polystyrene) and Accurel PP (non-ionic polymeric adsorbant, 200–400 mesh polypropylene) were used for the immobilization of lipase. Crude Amano P-30 lipase from *Pseudomonas* sp. (30 g) was dissolved in 75 ml distilled water and centrifuged at 15000g for 15 min at 4° C to obtain a clear supernatant. Supernatant (25 ml) was added to 1.0 g each of resin previously washed three times with 25 ml methanol. Resins and enzyme solutions were gently agitated on a gyratory shaker at room temperature for 24 h. Adsorption of enzyme to each resin was checked periodically by determining the lipase activity and protein content remaining in the supernatant obtained after centrifugation of the enzyme-resin mixture. After 24 h, the slurry was filtered and the cake was washed with 400 ml distilled water. The resins containing immobilized enzyme were dried under vacuum for 24 h.

Crude Amano P-30 *Pseudomonas* sp. lipase was also immobilized on Accurel PP on a preparative scale. The enzyme (500 g) was dissolved in 3.0 l of distilled water and centrifuged at 15000g for 15 min at 40° C to obtain a clear supernatant. Accurel PP (50 g) previously washed with methanol was added to the enzyme solution and the mixture was agitated gently on a gyratory shaker at room temperature for 24 h. Periodically, samples were taken to check lipase activity and protein in the supernatant obtained after centrifugation. After 24 h, the enzyme and resin slurry was filtered and the cake was washed with 15 l distilled water. The resin containing immobilized enzyme was dried under vacuum at 25° C for 72 h.

Analytical methods. The esterification of **1** was monitored by gas chromatography (GC) with a flame ionization detector (FID) of reaction mixture samples using a Hewlett Packard (Palo Alto, Calif., USA) fused silica capillary column (cross-linked methyl silicone, 15 m long, 1.0-µm film thickness, 0.31 mm I.D., HP 190912-215) at 215° C oven temperature, 250° C injection temperature, and 250° C detection temperature. The retention time for **1** was 3.8 min and for the corresponding methyl ester was 3.1 min.

The optical purity of **1** was determined by treating the samples with thionyl chloride and reacting the acid chloride formed with D-2-octanol to yield the diastereomers (Jemal and Cohen, 1987). In the procedure used, 1 ml of reaction mixture filtrate (3 mg of **1**) was evaporated to remove the solvent. One ml of 10% thionyl chloride solution in *n*-hexane containing 2.5 µl dimethylformamide was added to the vial, mixed thoroughly on a vortex mixer and incubated at room temperature for at least 30 min. The reagent was subsequently evaporated under a gentle stream of nitrogen at 50° C. To the dried residue, 0.3 ml of D-2-octanol was added, mixed thoroughly on a vortex mixer and incubated at 60° C for 30 min in a heating block. Octanol was evaporated under a stream of nitrogen at 50° C and the residue was dissolved in 0.5 ml methylene chloride. Diastereomers were analyzed by GC (FID) using a Hewlett Packard fused silica capillary column (HP no. 190912-215) at 215° C oven temperature, 250° C injection temperature, and 250° C detector temperature. The retention times for the esters of the L- and D-enantiomers were 14.8 min and 16.0 min, respectively.

Enzymatic reaction. The enzymatic reaction mixture for the esterification contained 0.1 M (22.4 mg/ml) of **1**, 0.4 M methanol, 0.1% water, and 1 g crude lipase in 10 ml toluene. The reaction was conducted at 40° C and 280 rpm. Periodically, samples were taken and analyzed by GC to determine the yield and optical purity of the product.

Evaluation of immobilized Amano P-30 lipase. Immobilized crude Amano P-30 lipase on Accurel PP was evaluated in a 3-ml and 50-ml reactor volume. The reaction mixture contained 0.1 M 3-benzoylthio-2-methylpropanoic acid (22.4 mg/ml), 0.4 M methanol, 0.1% water and 0.3 g and 6 g of immobilized enzyme in 3 ml or 50 ml toluene, respectively. The reaction was conducted at 28° C and 280 rpm.

Lipase fermentation. Pseudomonas sp. ATCC 21808 was used in the fermentation. One frozen vial of culture was used to inoculate 100-ml of F7 medium (1% w/v malt extract, 1% w/v yeast extract, 0.3% w/v peptone and 2% w/v glucose) in a 500-ml erlenmeyer flask. The flask was incubated at 28° C and 280 rpm for 72 h and the entire contents used to inoculate 1.5 l of F7 medium in a 4-l flask. The flask was incubated at 28° C and 180 rpm for 24 h before being used to inoculate the seed stage in a 380-l tank. The seed stage contained 150 l of medium L (3% w/v cornsteep liquor, 1.1% w/v cerelose hydrate, 0.6% w/v urea, 0.2% w/v potassium dihydrogen phosphate, 0.05% w/v potassium chloride, 0.05% w/v magnesium sulfate, 1% w/v soybean oil and 0.1% w/v antifoam). The culture was grown at 280 rpm agitation, 150 l/min aeration, 10 psig pressure and 26° C temperature. Seed culture (12.5 l) was used to inoculate 250 l of medium L contained in a 380-l fermentor. The fermentation conditions were 250 rpm agitation, 250 l/min aeration, 10 psig pressure, and 26° C.

Enzyme recovery. At the end of the fermentation, the culture broth was centrifuged through a high-speed Sharples centrifuge to remove cells. The filtrate was concentrated under vacuum at 50° C to one-tenth volume in a climbing film evaporator. Cold ethanol (250 l) at 5–10° C was added to 25 l stirred concentrate and the mixture was kept for 12 h at 0–5° C without agitation. The supernatant was decanted and the slurry was filtered. The filtered precipitate was washed with cold ethanol and the cake was vacuum-dried at 40° C for 48 h to obtain the crude lipase. This was immobilized on Accurel PP as described earlier.

Results and discussion

Screening of lipases

Commercially available lipases were screened for the stereoselective esterification of racemic **1** to yield the acid *R*-(+) methyl ester and unreacted acid enriched in the desired *S*-(−) **1**. Lipases (P-30, AK, CES-30, B1) from various *Pseudomonas* sp. catalyzed the stereoselective esterification (Table 1). *S*-(−) **1** was obtained with a reaction yield of 37% (based on racemic substrate initially present) and an optical purity of 97.2% using Amano Lipase P-30. Poor selectivity was observed with lipases from *C. cylindracea*, *Rhizopus* sp., *Mucor* sp., *Penicillium* sp. and *H. langinosa*. Further research was conducted using lipase P-30.

Table 1. Evaluation of lipases for the stereoselective esterification of 3-benzylthio-2-methyl propanoic acid (**1**)

Lipase source	Reaction time (h)	Conversion (%)	Yield (%)	Optical purity (%)
Candida cylindracea (Sigma Chem.)	1.3	64	36	38.7
Rhizopus sp. (Amano N)	26.5	58	42	56.0
Mucor sp. (Amano MAP)	0.5	78	22	58.4
Penicillum sp. (Amano P)	99.0	61	39	42.0
Pseudomonas sp. (Biocatalyst)	27.0	68	32	95.9
Pseudomonas sp. (Enzymatics)	27.0	58	42	91.7
Aspergillus niger (Amano APF)	99.0	86	14	68.6
Pseudomonas sp. (Amano AK)	27.0	56	44	86.4
Pseudomonas sp. (Amano CES)	27.0	59	41	82.6
Humicola langinosa (Amano CE)	27.0	78	22	56.1
Pseudomonas sp. (Amano P-30)	27.0	62	38	97.0

The reaction mixture in 5 ml toluene contained 0.025 M of **1**, 0.1 M methanol, 0.1% water, and 2 g crude lipase. The reaction was conducted at 40° C. The reaction yield and optical purities were determined by gas chromatography

Screening of alcohols

Six different alcohols were screened in the esterification reaction using lipase P-30 (Table 2). Methanol exhibited the highest stereoselectivity. Benzyl alcohol and 1-octanol also exhibited comparable stereoselectivity. Trifluoroethanol and 2-amino-1-butanol exhibited poor selectivity.

Immobilization and evaluation of lipase P-30

Crude lipase P-30 was immobilized on three different resins XAD-7, XAD-2 and Accurel PP. The absorption efficiencies were about 68%, 71%, and 98.5%, respectively (Table 3). These immobilized lipases were evaluated for the ability to stereoselectively esterify racemic **1**. As shown in Table 4, enzyme immobilized on Ac-

Table 2. Screening of alcohols for the esterification reaction

Alcohol	Reaction time (h)	Conversion (%)	Yield (%)	Optical purity (%)
Methanol	112	50.3	49.7	91.7
	136	55.1	44.9	95.4
	162	63.0	37.0	97.3
2-Amino-1-ethanol	16	68.3	31.7	50.0
	40	93.3	6.7	49.9
Trifluoroethanol	162	28.8	71.2	61.8
	328	38.4	61.6	63.0
Isopropylidene glycerol	112	33.0	67.0	68.6
	280	58.3	41.7	86.7
Benzyl alcohol	112	39.1	60.9	71.9
	280	66.9	33.1	96.0
1-Octanol	112	34.0	66.0	68.0
	280	68.0	32.0	95.1

The reaction mixture in 25 ml toluene contained 0.1 M (22.4 mg/ml) of **1**, 0.4 M alcohol as indicated, 0.1% water, and 1 g Amano lipase P-30. The reaction was conducted at 28° C and 280 rpm on a rotary shaker

Table 3. Immobilization of Amano P-30 lipase on various supports

Support	Adsorption time (h)	Lipase activity in filtrate (units/ml)	Protein (mg/ml)	Adsorption efficiency (%)
XAD-7	0	3060	103	0
	1	2035	80	33
	2	1890	66	38
	3	1770	64	42
	24	960	60	68
XAD-2	0	3950		0
	24	1145		71
Accurel PP	0	3950		0
	24	60		99

Crude Amano P-30 lipase (10 g) was immobilized on various supports (1.3 g each). The enzyme-support mixture was incubated at 28° C and 100 rpm. Periodically and after 24 h incubation, the immobilized enzyme was recovered by filtration and the filtrate was analyzed for lipase activity. Immobilized enzyme was dried in a vacuum oven at room temperature

curel PP catalyzed efficient esterification, giving 36–45% reaction yield and 97.7% optical purity of *S*-(−) **1**. The enzyme under identical conditions gave similar optical purity and yield of product in two additional reaction cycles.

Immobilized enzyme was evaluated for reusability in 3-ml and preparative 50-ml reactor volumes. The enzyme was reused over 23 cycles without loss of activity in a 3-ml reactor and over 15 cycles in a 50-ml reactor. The optical purity of 97.7% and reaction yield of 40% was obtained for *S*-(−) **1**.

Evaluation of solvents for esterification

Various solvents such as heptane, cyclohexane, *n*-octane and toluene were used in the esterification reaction. Toluene was the best based on the yield and optical purity of the product. Octane and cyclohexane gave products with high optical purities but in lower yields (Table 5).

Table 4. Evaluation of immobilized lipase P-30 on various supports for the esterification reaction

Cycle no.	Immobilized lipase on support	Reaction time (h)	Conversion (%)	Yield (%)	Optical purity (%)
1	XAD-7	17.5	70.6	29.4	96.7
	XAD-2	17.5	28.0	72.0	—
		25.0	35.5	64.5	—
		41.5	50.0	50.0	82.5
	Accurel PP	17.5	64.1	35.9	97.5
		25.0	66.1	33.1	97.7
2	XAD-7	17.0	61.2	33.1	91.8
	Accurel PP	17.0	49.1	50.9	—
		22.5	54.2	45.8	97.7
3	XAD-7	16.0	62.8	37.2	90.2
		16.0	55.6	46.4	96.7

The reaction mixture in 3 ml toluene contained 0.1 M substrate, 0.4 M methanol, 0.1% (v/v) water, and 0.3 g immobilized Amano P-30 lipase. The reaction was conducted at 28° C

Table 5. Effect of organic solvents on the esterification reaction

Solvent	Reaction time (h)	Conversion (%)	Yield (%)	Optical purity (%)
Heptane	16.5	82.9	17.1	92.2
Cyclohexane	16.5	90.6	9.4	97.8
N-Octane	16.5	91.4	8.6	97.3
Toluene	16.5	63.0	37.0	97.2

The reaction mixture in 4 ml solvent contained 0.1 M substrate, 0.4 M methanol, 0.1% (v/v) water, and 0.48 g immobilized Amano P-30 lipase. The reactions were conducted at 40° C and 280 rpm

Effect of Temperature on esterification

The esterification was conducted at 28° C, 40° C, and 60° C. On increasing the reaction temperature from 28° C to 40° C and to 60° C, the reaction time decreased from 18 h to 4 h and to 2 h, respectively. The optical purity (ca. 82%) obtained at 40° C and 60° C was inferior; however, a higher yield (46%) was obtained. The reaction yield of 37% and optical purity of 97.7% was obtained at 28° C.

Effect of substrate concentration on esterification

The racemic acid was evaluated at 0.25 M, 0.5 M and 1.0 M substrate concentrations with 1 M, 2 M and 4 M methanol, respectively, in the esterification reaction using 0.3 g lipase P-30 immobilized on Accurel PP. The yield (38–47%) was similar; however, the optical purity

Table 6. Effect of substrate concentration on the esterification reaction

Substrate concentration (M)	Methanol concentration (M)	Reaction time (h)	Conversion (%)	Yield (%)	Optical purity (%)
0.10	0.4	6.0	55.7	44.3	97.1
0.25	1.0	17.0	61.7	38.3	96.5
0.50	2.0	23.5	53.8	46.2	92.0
1.00	4.0	48.0	52.6	47.4	90.0

The reaction mixture in 10 ml toluene contained substrate as indicated, 0.6 M methanol, 0.1% (v/v) water, and 1.2 g Amano P-30 lipase immobilized on Accurel PP. The reaction was conducted at 40° C

Table 7. Effect of methanol concentration on the esterification reaction

Methanol concentration (M)	Reaction time (h)	Conversion (%)	Yield (%)	Optical purity (%)
0.5	5.5	55.0	45.0	93.6
1.0	8.0	61.2	38.8	96.0
1.5	21.0	60.0	40.0	96.5
2.0	30.0	62.0	38.0	95.5
3.0	64.0	13.0	87.0	56.0
4.0	64.0	6.0	94.0	56.0

The reaction mixture in 10 ml toluene contained 0.5 M substrate, 0.1% (v/v) water, and 1 g Amano P-30 lipase immobilized on Accurel PP. The reaction was conducted at 40° C

decreased with increasing substrate concentration (Table 6).

Effect of methanol concentration

Various methanol concentrations (0.5 M, 1.0 M, 2.0 M and 4.0 M), were evaluated at 0.5 M substrate concentration. The rate of esterification decreased as the methanol to substrate ratio was increased from 1:1 to 4:1 (Table 7). Higher methanol concentrations may inhibit the esterification reaction by stripping the essential water from the enzyme. Note the marked decrease in optical purity at >3 M methanol, indicating a marked change in the enzyme.

Lipase fermentations

The highest lipase activity achieved in batch fermentation using *Pseudomonas* sp. SC 13809 was 56 u/ml. A significant increase in lipase production was achieved using a fed-batch culture in which soybean oil was added to the fermentor at a level of 1% w/v. After 24 h of batch culture, additional soybean oil was supplied by adding 250 ml soybean oil every hour from 24 to 48 h.

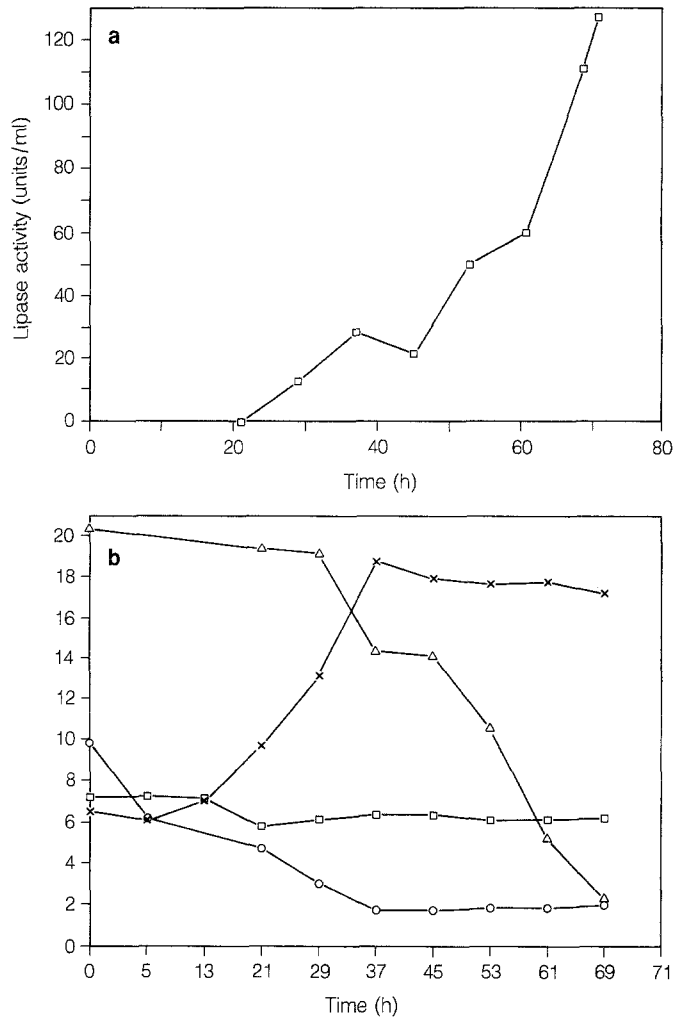


Fig. 2a, b. Lipase fermentation using *Pseudomonas* sp. ATCC 13809: □, pH; +, optical density (OD) at 600 nm; ◇, glucose (g/l); △, soybean oil × 10 (% w/w)

Table 8. Evaluation of Bristol-Myers Squibb lipase for the esterification reaction

Batch no.	Reaction time (h)	Conversion (%)	Yield (%)	Optical purity (%)
NWM-0589	68	55	45	80.7
NWM-0589, immobilized	39	52	48	87.7
NWM-0506	68	77	23	93.7
NWM-0889	39	65	35	96.5

Lipase fermentations were carried out using *Pseudomonas* sp. ATCC 13809 by a batch process (NWM-0589, NWM-0506) or fed-batch process (NWM-0889). Crude lipase recovered from the filtrate was used directly or immobilized on Accurel PP and used in the esterification reaction. The reaction mixture in 10 ml toluene contained 0.1 M substrate, 0.4 M methanol, 0.1% (v/v) water, and 2 g crude lipase or 0.5 g immobilized lipase

As seen in Fig. 2a, very little lipase was produced during the growth phase (up to 37 h). Most of the glucose was consumed during the first 37 h which corresponded to the period of rapid cell growth (Fig. 2b). Although lipase activity was low during the first 45 h of

fermentation, secretion of lipase quickly accelerated between 45 and 71 h of fermentation. The concentration of lipase, 128 units/ml, was increasing steeply at harvest and would undoubtedly have been greater had the fermentation been extended. The lipase activity achieved represents an increase of 129% over the highest we have previously achieved in a batch fermentation (56 units/ml).

Crude lipase (Bristol-Myers Squibb lipase) was recovered from the fermentation broth by ethanol precipitation and was also immobilized on Accurel PP. Crude lipase from three different fermentation batches (NWM0589, 0506 and 0889) and immobilized lipase from batch NWM0589 were evaluated in the esterification reaction (Table 8). A yield of 35% and optical purity of 96.5% were obtained using crude lipase (batch NWM-0889), while a yield of 48% and optical purity of 87.7% were obtained with immobilized lipase (batch NWM0589).

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