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Biotransformation of the Diphenyl Ether Herbicide Lactofen and Purification of a Lactofen Esterase from *Brevundimonas* sp. LY-2

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The diphenyl ether herbicide lactofen is commonly used to control broadleaf weeds. Once released into the environment, this herbicide is subject to microbial reactions. This study describes the biotransformation of lactofen by *Brevundimonas* sp. LY-2 isolated from enrichment cultures inoculated with soil sample. This strain degraded about 80% of 50 mg L^{-1} lactofen in 5 days of incubation in flasks. The metabolic behaviors of the herbicide in the media are described. The results show a transformation pathway of lactofen by the bacterium leading to the formation of 1-(carboxy)ethyl-5-(2-chloro-4-(trifluoromethyl)phenoxy)-2-nitrobenzoate and ethanol. An esterase, which could cleave the right ester bond of the alkanoic side chain of lactofen, was purified 113.3-fold to homogeneity with 6.83% recovery. The current results suggested that *Brevundimonas* sp. LY-2 degraded lactofen via the ester bond cleavage catalyzed by esterase.

KEYWORDS: Biotransformation; lactofen; intermediate metabolites; *Brevundimonas* sp.; esterase

INTRODUCTION

Lactofen is a member of the diphenylether herbicides. The major target of this class of herbicides is protoporphyrinogen oxidase in the porphyrin biosynthetic pathway (*1*, *2*). Lactofen has high activity and is commonly used to control broadleaf weeds in soybeans, cereal crops, potatoes, and peanuts (*3*). As a postemergent herbicide, it is directly released to the environment, and the environmental fate of lactofen has been studied (*4*, *5*). Hydrolysis is expected to be an important process under alkaline conditions based on an estimated hydrolysis half-life of 37 days at pH 8. Photolysis is thought to be minimal, and the photodegradation half-life of lactofen was reported as 24 days (*6*). This herbicide is degraded in soil mainly by microbial activity, and aerobic conditions speed the rate of microbial breakdown of lactofen (*7*). The biodegradation half-life of lactofen applied to a clay loam at an application rate of 5 mg kg^{-1} and maintained under anaerobic conditions was 30 days. The biodegradation products of lactofen in soil had been reported as acifluorfen and 1-(carboxy)ethyl 5-(2-chloro-4-(trifluoromethyl)phenoxy)-2-nitrobenzoate (*8*). The enantioselectivity of the transformation of lactofen also had been studied (*9*).

Currently, few reports on the microbial degradation of diphenyl ethers have been published. *Sphingomonas* sp. strain SS3 could utilize diphenyl ether and its 4-fluoro, 4-chloro, and 4-bromo derivatives as sole sources of carbon and energy (*10*). *Azotobacter chroococcum* could utilize herbicide oxyfluorfen as the sole carbon source for growth (*11*). Keum et al. (*12*) reported *Sphingomonas wittichii* RW1 could degrade chlomethoxyfen, nitrofen, and oxyfluorfen. The metabolism pathways of the microbial degradation of several diphenyl ethers also have been studied. *Trichosporon beigelii* SBUG 752 was able to transform diphenyl ether, and several oxidation products were identified (*13*). *Sphingomonas paucimobilis* could metabolize diclofop-methyl to diclofop acid and degraded diclofop acid to 4-(2,4-dichlorophenoxy)-phenol and 2,4-dichlorophenol and/or phenol (*14*). Keum et al. (*12*) found that nitrodiphenyl ethers could be degraded by *S. wittichii* RW1 through the initial reduction and N-acetylation of nitro groups, followed by ether bond cleavage. *Lysinibacillus* sp. ZB-1 could degrade fomesafen by reduction, acetylation of nitro groups, and dechlorination (*15*).

Up to now, little is known about the biotransformation of lactofen by pure culture. In this paper, a potent lactofen degrader was isolated from soils through repetitive enrichment and successive subculture. We also describe the transformation of lactofen by a newly selected bacterium together with identification of the corresponding metabolites and the purification of a lactofen esterase from cellfree extracts of this strain.

MATERIALS AND METHODS

Soil, Chemicals, and Media. Soil samples were collected from an agricultural field, which had been exposed to lactofen for many years, in the city of Qiqihaer, Heilongjiang Province, China. Lactofen (99% purity) was purchased from Sigma-Aldrich Chemical Co. (Shanghai, China). All other chemicals used in this study were of analytical grade. The Luria−Bertani medium contained yeast extract (5.0 g L^{-1}), tryptone (10.0 g L^{-1}), and NaCl (10.0 g L^{-1}), pH 7.0. Mineral salts medium (MM) contained NH_4NO_3 (1.0 g L^{-1}), K_2HPO_4 (1.5 g L^{-1}), KH_2PO_4 (0.5 g L^{-1}), NaCl (0.5 g L^{-1}), $MgSO_4$ (0.2 g L^{-1}), and 10 mL of a trace element solution, pH 7.0 (*16*). Solid medium plates were prepared by adding 1.5 wt %/vol agar into the above liquid medium. Lactofen dissolved in acetone was added to the media at a final concentration of 50 mg L^{-1}.

Isolation and Identification of the Strain. Soil samples (10.0 g) were added to 100 mL of MM with 50 mg L^{-1} lactofen and incubated at 30 °C in

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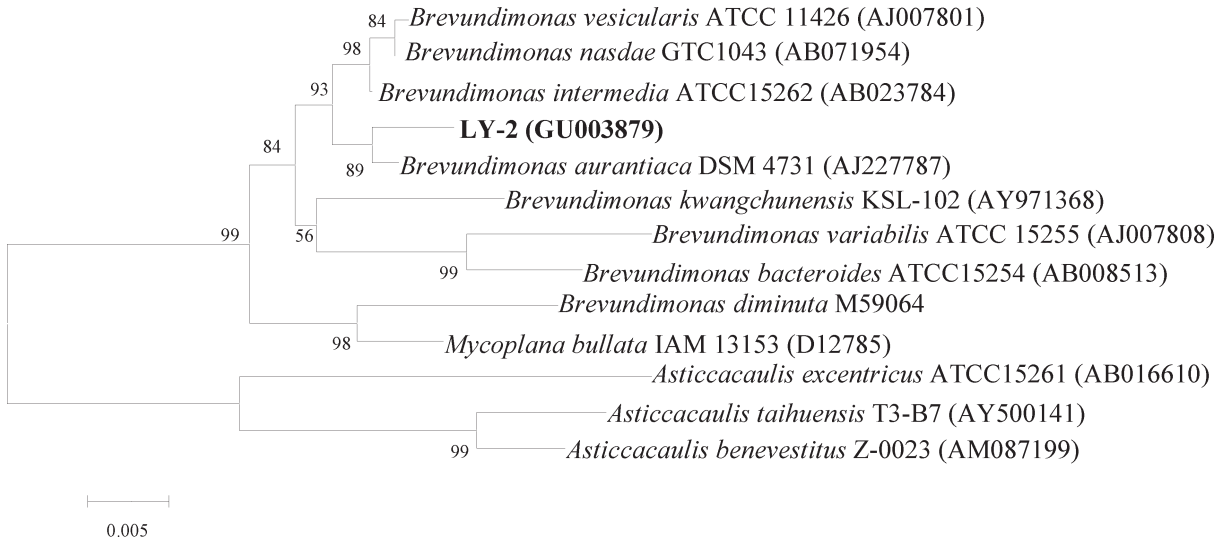

Figure 1. Phylogenetic tree for strain LY-2 and related species based on the 16S rRNA gene sequences.

a rotary shaker at 160 rpm for about 7 days. About 5 mL of enrichment culture was subcultured into 100 mL of fresh enrichment medium weekly. After six rounds of transfer, the loss of lactofen was measured by HPLC (as described below). Dilutions of the sequential enrichment were plated onto MM agar plates containing 50 mg L^{-1} lactofen. After incubation at 30 °C for 2 days, the colonies were purified and selected to verify their degrading capabilities. One pure isolate with lactofen degradation efficiency was isolated (designated LY-2) and selected for further investigation.

The identification of the strain was carried out by standard laboratory procedures and those described in *Bergey's Manual of Determinative Bacteriology* (*17*). The 16S rRNA gene was amplified by PCR using the following primers: 5′-AGAGTTTGATCCTGGCTCAG-3′ as forward and 5′-TACGGTTACCTTGTTACGACTT-3′ as reverse (*18*). This sequence was compared to known sequences found in the GenBank database using BLAST. Phylogenesis was analyzed with MEGA version 3.0 software, and its distance was calculated using the Kimura two-parameter distance model (*19*). Unrooted trees were built using the neighbor-joining method. Each data set was bootstrapped 1000 times.

Degradation of Lactofen by Strain LY-2 in Liquid Culture. Strain LY-2 was grown in LB medium. Cell culture was harvested in the late-exponential growth phase by centrifugation at 5000*g* for 5 min and washed twice with fresh MM medium. After the optical density at 600 nm (OD_{600}) had been adjusted to 1.0, an inoculum (2%, vol/vol) was inoculated into 50 mL of MM medium with the lactofen (50 mg L^{-1}). The cultures were incubated at 30 °C and 160 rpm. At regular time intervals, aliquot samples were collected, and the concentration of the herbicide was determined by HPLC. During the degradation, two products appeared and were analyzed by LC-MS and GC, respectively. Each treatment was performed in three replicates, and the control experiment with autoclaved microorganism was carried out under the same conditions.

Preparation of Cellfree Extracts. Cells in LB liquid medium were harvested by centrifugation (5000*g*, 5 min) at 4 °C, washed twice with Tris-HCl buffer (pH 7.2), and resuspended in the same buffer (20 mL) at a concentration equivalent to an OD_{600} of 5.0. The cells were ruptured by 3 s pulsed sonication for 10 min. The disrupted cell suspension was centrifuged at 12000*g* for 20 min at 4 °C to remove cell debris and undisrupted cells. The supernatant was passed through a cellulose acetate filter with a pore size of 0.2 mm. The microbial extract was suspended in Tris-HCl buffer, pH 7.2. Protein concentration was quantified according to the method of Bradford (*20*). Bovine serum albumin (Sigma) was used as standard for calibration.

Protein Purification. All of the following experiments were carried out at 4 °C unless otherwise specified. The cellfree extract of strain LY-2 produced a transparent halo of lactofen degradation on the phosphate buffered saline solid plates containing 200 mg L^{-1} lactofen within 1 h of incubation at 30 °C, which was utilized as an indicator to monitor the lactofen-degrading activities. Solid ammonium sulfate was added, with continuous stirring, to the enzyme suspension at 0−20% saturation and the resulting suspension settled for 20 min. The precipitate formed was removed by centrifugation (10000*g*, 15 min). The resulting supernatant was subjected to further precipitation by ammonium sulfate at saturations of 20−40, 40−60, and 60−100%. All fractions were desalted by dialysis and then concentrated. The esterase was purified using anion exchange column chromatography. The sample was applied to a DEAE Sepharose Fast Flow column (1.6 cm × 8 cm, GE Healthcare Bio-Sciences AB), which was pre-equilibrated with 20 mM Tris-HCl, pH 7.2. The column was initially eluted with 2 bed volumes of the buffer to wash out unabsorbed proteins. The adsorbed proteins were then eluted with a linear gradient of 0−250 mM NaCl in Tris-HCl, pH 7.2, buffer at a flow rate of 0.5 mL min^{-1}. The gradient eluate was collected in 2.5 mL fractions. Protein fractions were collected and monitored for esterase activity as described above. Those fractions containing esterase activity were combined and stored at −20 °C. The concentrated enzyme solution was subjected to native polyacrylamide gel electrophoresis (PAGE) as described by Waterborg and Matthews (*21*). When the PAGE completed, the polyacrylamide gel was transferred onto a 200 mg L^{-1} lactofen agar plate and incubated for 60 min at 30 °C. Then a clear transparent band appeared on the plate due to the degradation of the lactofen. The polyacrylamide gel slice corresponding to the transparent band was excised with a scalpel. The separation of the lactofen-degrading enzyme from the gel was achieved using a D-tube Electroelution Accessory Kit (Novagen).

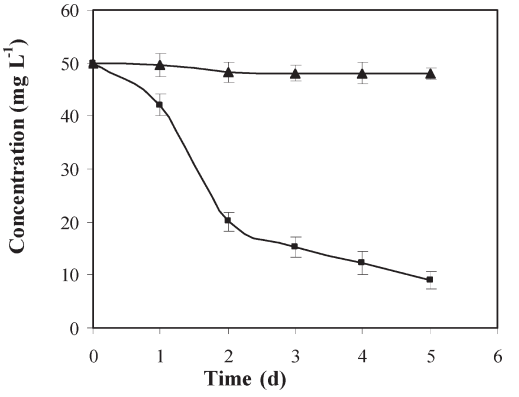

Figure 2. Biodegradation of lactofen by *Brevundimonas* sp. LY-2: (▲) autoclaved LY-2; (■) active LY-2. Error bars represent the standard error of three replicates.

Determination of Esterase Activity. Esterase activity was measured by adding 20 μL of enzyme preparation to 2 mL of 20 mM Tris-HCl, pH 7.2, containing 50 mg L^{-1} lactofen and incubating for 30 min at 30 °C. Samples were extracted, and the lactofen residues were measured by HPLC. The transformation products by esterase were identified by LC-MS and GC, respectively. Control samples containing boiled extract were treated and analyzed in the same way. All experiments were carried out in

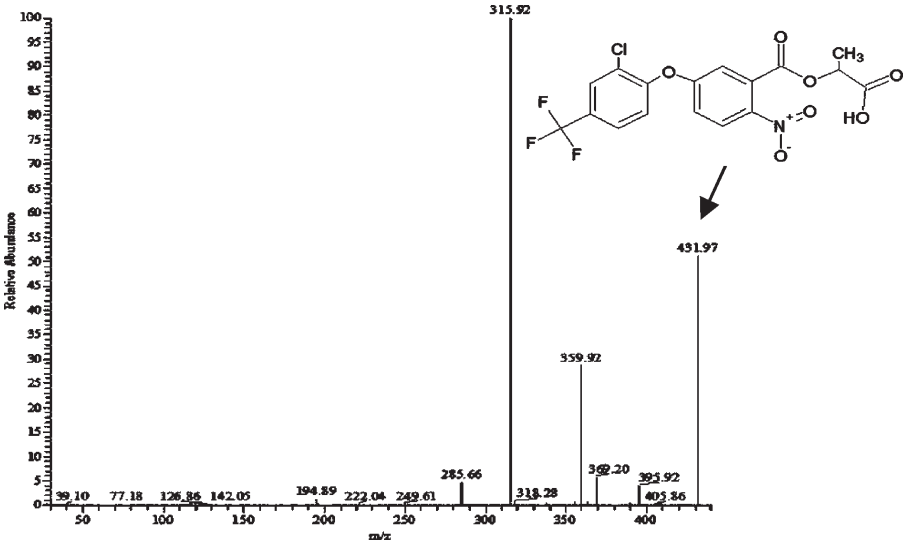

Figure 3. Mass spectrum of metabolite product A.

triplicate. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of ethanol from the substrate with concentration of 50 mg L^{-1} per minute at 30 °C. The molecular mass of the denatured protein was determined by SDS-PAGE (*22*). The molecular mass markers used were rabbit muscle phosphorylase *b* (97.2 kDa), bovine serum albumin (66.4 kDa), hen egg white ovalbumin (44.2 kDa), bovine carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa), and hen egg lysozyme (14.3 kDa). To determine the optimum pH, all activities were measured using 20 mM citric acid−NaOH buffer at pH 4.0−5.5, 20 mM phosphate buffer at pH 5.0−8.0, 20 mM Tris-HCl buffer at pH 7.2−9.0, and 20 mM glycine−NaOH buffer at pH 8.5−10.0. The optimum temperature was determined analogously with a constant pH of 7.0 and different temperatures from 10 to 80 °C. The kinetic parameter study was measured at the same conditions used for esterase activity determination by adding 20 μL of enzyme preparation to 2 mL of 20 mM Tris-HCl, pH 7.2, containing 0.1−100 μM lactofen and incubating for 30 min at 30 °C.

Chemical Analysis. Lactofen in liquid cultures were extracted with the same volume of dichloromethane and dried over anhydrous sodium sulfate. The mixture was warmed in a hot water bath to evaporate the dichloromethane. The residues were dissolved in acetonitrile. All samples were analyzed by HPLC equipped with a Zorbax C-18 ODS Spherex column (250 mm × 4.6 mm). The mobile phase was acetonitrile/water (75:25, v/v), and the flow rate was 0.8 mL min^{-1}. Detection of lactofen was recorded at 230 nm. The injection volume was 20 μL.

The MS apparatus was an LC-MSD-Trap-SL equipped with an electrospray ionization source and operated in the negative polarity mode. The ES-MS interface was operated under the condition of a gas temperature of 350 °C and a drying gas flow of 9.0 L min^{-1}. The nebulizer nitrogen gas pressure was 45 psi. Full scan signals were recorded within the *m*/*z* range from 50 to 1000. For LC-MS, the source parameter was spray voltage = 7.0 kV. The sheath and auxiliary gases were supplied with nitrogen.

For metabolic product ethanol from liquid culture, 1 mL of culture was collected from the medium and centrifuged at 12000*g* for 5 min and filtered through a hydrophilic polyether sulfone (PES) syringe filter. The analysis of ethanol was conducted by gas chromatography coupled with a flame ionization detector (FID) (Shimadzu GC-2010, Japan), a 30 m Rtx-Wax capillary column (0.25 mm id, film thickness = 0.25 μm), and a flame ionizing detector (H_2, 30 mL min^{-1}; air, 250 mL min^{-1}). Nitrogen was used as the carrier gas (20 mL min^{-1}). The temperatures at the injector, detector, and oven were set at 210, 250, and 280 °C, respectively. The injection volume was 0.5 μL. Standard solutions of ethanol (in 0.1% w/v) were mixed with MM medium as following concentrations (mg L^{-1}): 0, 0.5, 1, 2, 4, 8.

Recovery Assay. Triplicate analyses were conducted with liquid culture at levels of 0.1, 1.0, 10.0, and 50 mg L^{-1} lactofen. Extraction and analysis were performed as described above. The recoveries of lactofen from liquid culture at levels of 0.1, 1.0, 10.0, and 50 mg L^{-1} were determined to be 87.91 ± 6.2, 90.12 ± 4.3, 96.89 ± 2.7, and 99.12 ± 0.8%, which indicated that the procedure was efficient in extracting lactofen from liquid culture.

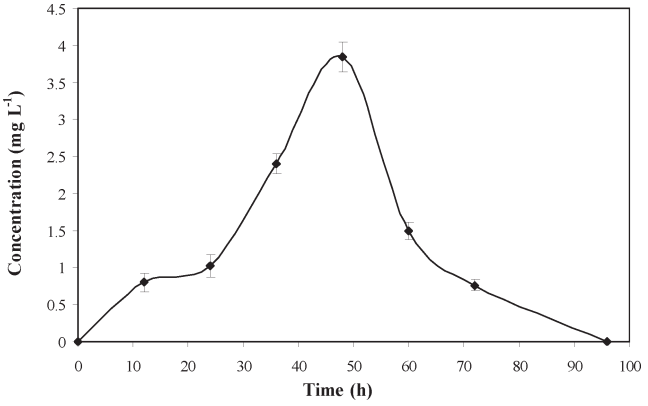

Figure 4. Time course of ethanol (product B) produced by *Brevundimonas* sp. LY-2. Error bars represent the standard error of three replicates.

RESULTS AND DISCUSSION

Strain Isolation and Identification. Strain LY-2, capable of degrading lactofen, was isolated and selected for further study due to its degrading efficiency. This strain forms smooth, circular, yellow pigmented colonies on LB plates. Strain LY-2 is Gram-negative with rod-shaped morphology. It tested positive for starch hydrolysis, catalase, and oxidase, but negative for glutin hydrolysis, Vogese Proskauer, and nitrate reduction. The sequence of the 16S rRNA gene of LY-2 was deposited at GenBank under accession number GU003879. The phylogenetic tree of the 16S rRNA sequence is shown in **Figure 1**, and this sequence was 99% identical to that of the 16S rRNA gene of *Brevundimonas aurantiaca* DSM 4731(*23*). According to its physiological characteristics and 16S rRNA phylogenetic analysis, strain LY-2 could be identified as a *Brevundimonas* sp.

Few strains of this genus with the ability to metabolize compounds have been isolated. Deshpande et al. (*24*) reported that the organophosphorus insecticide dimethoate could be degraded by *Brevundimonas* sp. MCM B-427. Herbicide 4-(2,4-dichlorophenoxy)-butyric acid and 4-(4-chloro-2-methylphenoxy)butyric acid degrading communities have been isolated, including *Brevundimonas* sp. (*25*). In our study, strain *Brevundimonas* sp. LY-2 was capable of degrading lactofen, which was another documented strain of *Brevundimonas* species with the ability to degrade xenobiotics.

Biodegradation of Lactofen by Strain LY-2. The time course curve of lactofen degradation by strain LY-2 is presented in **Figure 2**.

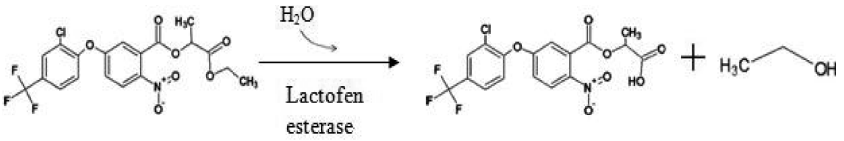

Figure 5. Proposed metabolic pathway of lactofen degraded by *Brevundimonas* sp. LY-2.

Table 1. Purification of the Lactofen Esterase

purification step	total activity (U)	total protein (mg)	specific activity (U mg^{-1})	purification fold	recovery (%)
cellfree crude enzyme	78.5	183.2 ± 6.7	0.43 ± 0.023	1	100
ammonium sulfate precipitation	52.1	76.32 ± 1.1	0.68 ± 0.03	1.58	66.37
ion-exchange chromatography	41.2	7.3 ± 0.33	5.64 ± 0.21	13.1	52.5
PAGE gel electronic elution	5.36	0.11 ± 0.005	48.73 ± 0.98	113.3	6.83

HPLC analysis showed a substantial reduction in the levels of lactofen. After incubation for 5 days, about 81.8% of 50 mg L^{-1} lactofen initially added to the MM medium was degraded by strain LY-2. This strain was deposited in the China General Microbiological Culture Collection Center under accession number CGMCC3651.

Up to now, several reports about the biodegradation of diphenyl ether herbicides have been described. Chakraborty et al. (*11*) isolated *A. chroococcum*, which could utilize oxyfluorfen as the sole carbon source and degrade >60% of added oxyfluorfen at a concentration of 250 mg L^{-1} in 7 days. Adkins (*14*) reported that 1.5 mg L^{-1} diclofop-methyl could be degraded by *S. paucimobilis* within 54 h. Chlomethoxyfen, oxyfluorfen, and nitrofen were degraded by *S. wittichii* RW1 after 7 days of incubation in the nutrient broth-supplimented M9 medium (*12*).

Identification of the Metabolites. The metabolites produced by the degradation of lactofen were extracted and identified by LC-MS and GC, respectively. One degradation product (designated product A) appeared during biodegradation of lactofen in MM medium at 12 h, and its concentration reached the highest level at 48 h followed by a decrease with longer incubation times. At 96 h, product A disappeared. The negative-ion chemical ionization of product A (retention time = 4.463) showed a prominent protonated molecular ion at m/z 431.97 $[M - H]^-$ and characteristic fragment ion peaks at m/z 359.92 $[M - COOH - C_2H_4 - H]^-$ and 315.92 $[M - COOH - C_2H_4 - CO_2 - H]^-$ (**Figure 3**). On the basis of these results, product A was preliminarily identified as 1-(carboxy)ethyl 5-(2-chloro-4-(trifluoromethyl)phenoxy)-2-nitrobenzoate, which was produced by hydrolysis of the right ester bond of the alkanoic side chain of lactofen. Product A has been reported as a degradation product of lactofen in soil (*8*). In this paper, product A was found during the degradation of lactofen by pure culture. Accompanying product A, another product (product B) appeared and has been detected by GC during degradation of lactofen in MM medium at 12 h. Product B was confirmed by the same retention time with ethanol authentic standard chemical in GC analysis, and it could be identified as ethanol. The time course of the production and disappearance of ethanol by strain LY-2 are shown in **Figure 4**. After an initial incubation period of 0−24 h, ethanol was accumulated dramatically, and the highest concentration of ethanol was observed at 48 h. With further incubation, its release declined and disappeared after 96 h. From the structures of the identified metabolites, one pathway is proposed for the biotransformation of lactofen by strain LY-2 (**Figure 5**).

Protein Purification. The lactofen esterase from cellfree extracts was purified using a procedure that involved ammonium sulfate precipitation, ion-exchange chromatography, and polyacrylamide gel electrophoretic elution (**Figure 6**). The summary of the purification is presented in **Table 1**. The enzyme was purified 113.3-fold to a specific activity of 48.73 U mg^{-1} of protein from the cellfree extract with recovery of 6.83% after the final step.

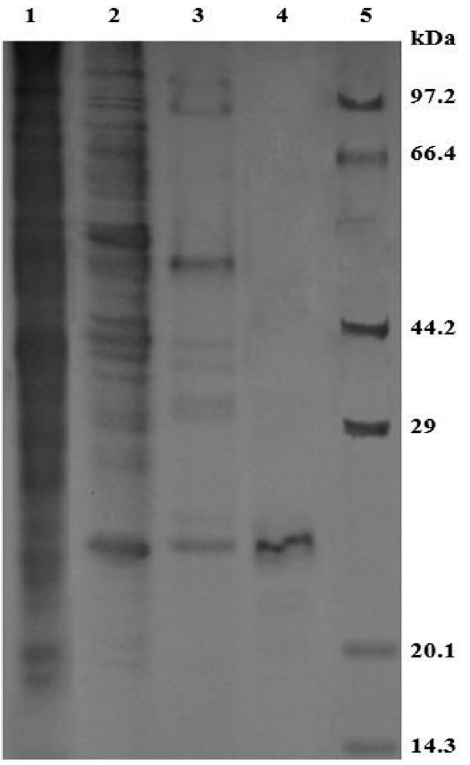

Figure 6. SDS-PAGE analysis of the purified esterase from *Brevundimonas* sp. LY-2. Lanes: 1, cellfree extract; 2, after ammonium sulfate precipitation; 3, after anion exchange column chromatography; 4, after PAGE gel electronic elution; 5, molecular mass markers.

The lactofen-degrading metabolite produced by the purified esterase was also identified and confirmed by LC-MS and GC. The purified enzyme catalyzed the hydrolysis of the right ester bond of the alkanoic side chain of lactofen to 1-(carboxy)ethyl 5-(2-chloro-4-(trifluoromethyl)phenoxy)-2-nitrobenzoate and ethanol. At 60 min, the concentration of ethanol reached the highest level, 4.75 mg L^{-1}, followed by stability for the rest of the incubation time. Therefore, the purified enzyme was designated a lactofen esterase. These degrading products were also found during the degradation of lactofen by strain LY-2 in liqulid culture (products A and B). When lactofen was transformed by the cells of strain LY-2, ethanol disappeared after a longer period of incubation, which showed that the strain could utilize ethanol for growth. However, when lactofen was hydrolyed by the purified esterase, the concentration of ethanol was stable after reaching the highest level, indicating the purified esterase only hydrolysis of the ester bond.

The molecular mass of esterase was found to be 25 ± 1 kDa as estimated by SDS-PAGE (**Figure 6**). The optimum pH and temperature for the enzyme were 7.0 and 20 °C, respectively. Measurement of esterase activity using lactofen concentrations ranging from 0.1 to 100 μM indicated that the K_m and V_{max} of the enzyme were 0.81 μM and 1.26 nmol min^{-1} mg^{-1}, respectively.

At present, many compound-degrading esterases have been studied. Esterase purified from *Gordonia* sp. strain MTCC 4818, which could utilize a number of phthalate esters as sole source of carbon and energy, is involved in the hydrolysis of butyl benzyl phthalate (*26*). An enzyme hydrolyzing ethylene glycol dibenzoate was purified from *Aspergillus nomius* HS-1 with a monomeric structure, of which the molecular mass was about 60000 (*27*). Three sulfonylurea herbicide pyrazosulfuron-ethyl degrading bacteria could produce extracellular carboxyesterase, which catalyzed the rapid de-esterification of *p*-nitrophenyl butyrate to *p*-nitrophenol (*28*). Pesticide pyrethroid-degrading esterases have been purified from *Bacillus cereus* SM3, *Aspergillus niger* ZD11, and *Klebsiella* sp. ZD112 (*29*−*31*). However, there is no report on the purification of esterase from pure strain, which could degrade diphenyl ether herbicides. In this paper, a lactofen esterase was purified from strain LY-2. To our knowledge, this is the first lactofen esterase purified to homogeneity from pure strain, and further genetic studies may lead to the discovery of novel genes involved in the future.

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