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Endophytic Fungi Associated with Mediterranean Plants as a Source of Mycelium-Bound Lipases

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A screening of endophytic fungi isolated from Mediterranean plants rendered a mycelium-bound lipase from a strain of *Rhizopus oryzae* that catalyzed the esterification of fatty acids in isooctane. The influence of various factors (water content, temperature, and pH) on ester synthesis was investigated. Catalytic activity was inversely correlated with water content. This enzyme was active over the entire pH range studied, from pH 3 to pH 8, and activity was maximal at pH 4 and pH 7. The enzyme was thermostable, with maximal activity at 60 °C.

KEYWORDS: Endophytic fungi; mycelium-bound lipase; resting cells; esterification; lipase activity; *Rhizopus oryzae*

INTRODUCTION

Lipases (EC 3.1.1.3) are enzymes that hydrolyze the ester bonds of water insoluble substrates at the interface between the substrate and the water. This reaction is reversible, and lipases may catalyze ester synthesis and transesterification in reaction mixtures with low water contents. Lipases catalyze a wide diversity of reactions, which has led to their widespread use in industrial applications (*1*).

Lipases are produced by animals, plants, and microorganisms. Most of the lipases used in biotechnological applications to date were isolated from fungi (*2*, *3*). Commercial fungal lipases are generally extracellular enzymes, although there is evidence for considerable mycelium-bound lipase activity (*4*). The use of naturally bound lipases is potentially cost effective because the biomass can be used directly, thereby eliminating the need for isolation, purification, and immobilization procedures and minimizing the loss of enzyme activity. Naturally bound lipases also offer other advantages, such as greater stability in the presence of organic solvents, high temperatures, and extreme pH values (*5*).

Industrial demand for new sources of lipases is continuing to stimulate the isolation and screening of new lipolytic microorganisms. We focused our attention on endophytic fungi as a potential new source of lipases. The specific association of fungal endophytes with plant hosts represents a large unexplored domain ripe for discovery (*6*). The term endophyte is commonly used for fungi and bacteria that for all or part of their life cycle invade the tissues of living plants, causing unapparent and asymptomatic infections entirely within plant tissues but no symptoms of disease (*7*).

In this study, we screened endophytic fungi displaying lipaselike activity. We identified a mycelium-bound lipase activity in a *Rhizopus* isolate and studied the effects of various factors on this activity.

MATERIALS AND METHODS

Chemicals. Trifluoroacetic acid (99.5%) was purchased from Fluka. 1-Dodecanol (98%) and linoleic acid (99%) were purchased from Aldrich. 1-Propanol (99%) and potassium hydrogenphthalate ($KHC_8H_4O_4$) were obtained from Probus. Isooctane (99%) was obtained from Panreac. Oleic acid, potassium dihydrogenphosphate (KH_2PO_4), and tris(hydroxymethyl)aminomethane ($C_4H_{11}NO_3$) were purchased from Merck.

Plant Material. The plants used in this study are listed in **Table 1**. All samples were collected in October 2000 from The Montsec Natural Park in Catalonia (Spain).

Isolation of Endophytic Fungi. Healthy plant tissues were harvested for the isolation of endophytic fungi. The tissues were washed in running tap water and processed within 24 h of collection. From each washed plant, we cut six segments, each 2−3 cm long. All segments were surface-sterilized by sequential washes in 0.53% sodium hypochlorite (2 min) and 70% ethanol (2 min) and were then rinsed with sterile distilled water and allowed to surface dry under aseptic conditions (*8*, *9*). The surface-sterilized segments were placed on 2% malt extract agar in Petri dishes. All plates were incubated at room temperature for a maximum of 2 weeks. Fungi growing out from the plant tissues were transferred to mycological agar, composed of 10 g of glucose, 10 g of papaic digest of soybean meal, and 15 g of agar per liter of distilled water. The plates were incubated for 5−7 days at 30 °C.

Preliminary Screening of Lipolytic Activity of Fungi. *Preparation of Medium.* The medium used for preliminary screening consisted of 5.0 g of mycological peptone, 3.0 g of yeast extract, and 10.0 g of agar per liter of distilled water. The medium was autoclaved at 121 °C for 15 min. After the medium was cooled to 60 °C, filter-sterilized tributyrin was added to the medium to a final concentration of 1.0% (v/v) and the mixture was emulsified by blending for 1−3 min. The blended medium was dispensed into Petri dishes immediately after homogenization and chilled rapidly (*10*).

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Table 1. Number of Strains Isolated from Each Plant Species[a]

species	N	code	species	N	code
Lavandula latifolia	6	1A–1F	*Erinacea anthyllis*	3	15A–15C
Rosmarinus officinalis	5	2A–2E	*Ononis tridentata*	3	16A–16C
Salvia lavandulifolia	2	3A, 3B	*Psoralea bituminosa*	2	17A, 17B
Salvia verbenaca	1	4	*Juniperus oxycedrus*	4	18A–18D
Satureja montana	5	5A–5E	*Juniperus phoenicea*	6	19A–19F
Sideritis scordioides	3	6A, 6C	*Arctostaphylos uva-ursi*	2	20A, 20B
Teucrium polium	6	7A–7F	*Buxus sempervirens*	3	21A–21C
Thymus vulgaris	1	8	*Daphne gnidium*	2	22A, 22B
Thymus zygis	2	9A, 9B	*Gypsophila struthium*	2	23A, 23B
F. vulgare	5	10A–10E	*Limonium hibericum*	3	24A–24C
Seseli tortuosum	5	11A–11E	*Olea europaea*	5	25A–25E
Artemisia herba-alba	1	12	*Plantago albicans*	3	26A–26C
Jasonia glutinosa	2	13A, 13B	*Quercus ilex* subspecies *ballota*	6	27A–27F
Santolina chamaecyparissus	6	14A–14F			

[a] The identification code used is shown. *N* = number of strains isolated. Code: identification code for isolated strains.

Inoculation, Incubation, and Measurement of Clearing Area. The inoculum to be tested was cultured on mycological agar plates for 5−7 days at 30 °C. A disk of solid medium containing inoculum was excised from this plate with a sterile plunger and placed in the middle of an agar plate containing the substrate tributyrin. Plates were incubated at 25 °C for 5 days. The diameter (cm) of the cleared zone around the disk was used to estimate the relative lipolytic activities of the fungi tested.

Secondary Screening. We assessed the catalytic activity of the selected fungi by monitoring propyl oleate synthesis.

Culture of Microorganisms. Microorganisms were cultured in a synthetic liquid medium consisting of 2 g of asparagine, 2 g of glucose, 1 g of K_2HPO_4, 0.5 g of $MgSO_4$, 5 mg of thiamine hydrochloride, 1.45 mg of $Fe(NO_3)_3 \cdot 9H_2O$, 0.88 mg of $ZnSO_4 \cdot 7H_2O$, and 0.235 mg of $MnSO_4 \cdot H_2O$ per liter of distilled water (*11*). The pH of the medium was adjusted to 5.5−6.0. The medium was sterilized (121 °C for 15 min) in 250 mL aliquots, to which we added 2% (v/v) refined sunflower oil in aseptic conditions. The medium was inoculated with 2.5 mL of a spore suspension ($(1-4) \times 10^6$ spores mL^{-1}) for sporulating isolates or with four disks cut out from mycological agar for nonsporulating cultures. It was then incubated at 28 °C for 5 days on an orbital shaker (200 rpm).

Preparation of Resting Cells and Activity Assay. Mycelium was harvested from the culture medium with a Buchner funnel and was washed with distilled water and then with acetone. The mycelium was dried under vacuum for 18 h and ground to a powder (*12*).

To study the mycelium-bound lipase activity, 50 mg of lyophilized mycelium was suspended in 10 mL of a solution of 89 mM oleic acid and 666 mM 1-propanol in isooctane. This suspension was incubated for 3 h at 25 °C, with shaking at 200 rpm, on an orbital shaker. Samples (1 mL) were withdrawn and filtered. The amount of propyl oleate was quantified by gas chromatography, as described below. Experiments were carried out in triplicate.

Factors Affecting Mycelium-Bound Lipase Activity. *Growth Media and Culture Conditions.* Fungi were cultured as described for secondary screening, except that glucose was omitted from the culture medium (*12*).

Effect of Water Content on Lipase Activity. Lyophilized mycelia were dried under vacuum over P_2O_5 at room temperature for 2 h (*13*). Reactions were carried out immediately after drying to prevent the mycelium from taking up water. We added 5 mg of lyophilized mycelium to one vial and 5 mg of lyophilized and dried mycelium to another vial, each vial containing 4 mL of a solution of 11.1 mM linoleic acid and 22.2 mM 1-propanol in isooctane. The reaction mixtures were incubated at 26 °C for 5 min, with a magnetic stirrer turning at a speed of 500 rpm. Samples (1 mL) were withdrawn and filtered, and the resting cells that accumulated on the filter were allowed to dry. We suspended 5 mg of dried resting cells for each sample in several vials with 3 mL of isooctane and either 0, 5, 7, 10, 15, or 20 μL of water and incubated them for 15 h at 26 °C. We then added 1 mL of a solution of 44.5 mM linoleic acid and 89.0 mM 1-dodecanol in isooctane to each vial and incubated the reaction mixtures at 26 °C, with a magnetic stirrer turning at a speed of 500 rpm. Samples (1 mL) were withdrawn after 5 min and filtered. We monitored ester formation and residual free fatty acid levels by reverse phase high-performance liquid chromatography (RP-HPLC), as described below. Experiments were carried out in triplicate.

Effect of Temperature on Lipase Activity. To determine the effect of temperature, three vials containing 5 mg of lyophilized mycelium suspended in 3 mL of isooctane were prepared for each reaction time (5 min, 20 min, and 2 h) and each temperature (30, 40, 50, 60, 70, and 80 °C). Each vial was preincubated at the appropriate temperature for 30 min, with a magnetic stirrer turning at 500 rpm, before the start of the experiment. We then added 1 mL of a solution of 44.5 mM linoleic acid and 89.0 mM 1-dodecanol in isooctane, warmed to the same temperature, to each vial. The reaction mixtures were then stirred, with a magnetic stirrer turning at a speed of 500 rpm, and 1 mL aliquots were withdrawn at various reaction times and filtered. The amount of dodecyl linoleate was quantified by gas chromatography, as described below.

Thermostability of Mycelium-Bound Lipase. A set of vials, each containing 5 mg of lyophilized mycelium suspended in 3 mL of isooctane, was incubated at 60 or 70 °C for 2.5 h. The vials were cooled to 30 °C, and 1 mL of a solution of 44.5 mM linoleic acid and 89.0 M 1-dodecanol in isooctane, warmed to 30 °C, was added to each vial. The reaction mixtures were then incubated at 30 °C, with a magnetic stirrer turning at a speed of 500 rpm. Samples (1 mL) were withdrawn at various reactions times and filtered. The amount of dodecyl linoleate was quantified by gas chromatography, as described below. Experiments were carried out by triplicate.

Effect of pH on Lipase Activity. The effect of pH on the activity of mycelium-bound lipase was measured at several pH values. A potassium hydrogenphthalate ($KHC_8H_4O_4$) buffer was used to control pH in the 3−5.5 range. A potassium dihydrogenphosphate (KH_2PO_4) buffer was used to control pH in the 6−7.5 range. Finally, a tris(hydroxymethyl)-aminomethane ($C_4H_{11}NO_3$) buffer was used to control pH at 8. All the buffers were made up from stock solutions of 0.05 M salts using either 0.1 M NaOH or 0.1 M HCl to reach the required pH. The mycelium was first incubated in buffered solutions for 3 h at 26 °C. It was then filtered, washed three times with distilled water and three times with acetone, lyophilized for 2 h, and ground to a powder. To assay enzyme activity, we prepared three vials for each reaction time (5 and 20 min), each containing 4 mL of 11.1 mM linoleic acid and 22.2 mM 1-propanol in isooctane. The reaction mixtures were stirred with a magnetic stirrer turning at 500 rpm. Aliquots (1 mL) were withdrawn at different reaction times and filtered. The amount of propyl linoleate was quantified by gas chromatography, as described below.

Chromatographic Analyses. *HPLC Analysis.* HPLC analyses were carried out with a Waters series 600 pumping system, a Waters 710 autosampler, and a Waters 2690 UV detector at 210 nm (Waters Cromatografia SA, Spain). Twenty microliters of each sample was injected into a Simmetry C_{18} 5 μm (150 mm × 3.9 mm) (Waters Cromatografia SA) reverse-phase column at 40 °C. The eluent was 95% ethanol/5% 0.1% trifluoroacetic acid in Milli-Q water (95:5 v/v).

Table 2. Diameter of the Cleared Zone (Halo) after 5 Days of Incubation at 25 °C for Each Isolated Strain[a]

strain	*H*	strain	*H*	strain	*H*	strain	*H*	strain	*H*
1A	0.9	6C	1.1	11C	1.6	17B	1	23B	2.1
1B	1	6A		11D	1.6	18A	1.9	24A	1.9
1C	1.2	6B	1.4	11E	1.5	18B	2	24B	
1D	1.2	7A	1.6	12		18C	1.6	24C	1.8
1E	1.2	7B	1.2	13A	2.1	18D	1.9	25A	1.1
1F	1.3	7C	1.4	13B	1.8	19A	2.5	25B	1.1
2A	1.2	7D	1.3	14A	1.2	19B		25C	
2B		7E		14B	1.1	19C	1.6	25D	1.3
2C	1.1	7F	1.2	14C	1.3	19D		25E	0.9
2D	1.2	8	1.4	14D	1.2	19E	1.8	26A	1.1
2E	1.3	9A	1	14E	1.2	19F	1.7	26B	1.3
3A		9B	1.1	14F	1.2	20A	1.8	26C	1.9
3B	1.2	10A	8.6	15A		20B	1.7	27A	0.9
4		10B		15B	1.6	21A	1.8	27B	
5A	1.2	10C	2	15C	1.5	21B		27C	
5B	1.2	10D	1.8	16A	1.2	21C	1.6	27D	
5C	1.1	10E	1.8	16B	1.3	22A	1.9	27E	
5D	1	11A	1.6	16C	1.2	22B	1.6	27F	1.1
5E	1.2	11B	1.5	17A		23A	1.8		

[a] The identification code used is shown. *H* = halo diameter (cm).

The flow rate was 1 mL/min. Chromatographic peaks were recorded and integrated with Millenium 32 computer software (Waters Cromatografia).

GC Analysis. Samples were analyzed with a ThermoQuest series 2000 chromatograph equipped with an FID detector, an EEP system (Fisons, Barcelona, Spain), a split/splitless injection system, and an autosampler. The analytical column (Supelco, Madrid, Spain) was a 30 m × 0.25 mm fused silica capillary coated with a 0.25 μm film of poly(80% biscyanopropyl−20% cyanopropylphenyl siloxane) (SP-2330). The GC run was programmed such that the temperature increased from 50 to 150 °C (180 °C for dodecyl ester) at a rate of 5 °C/min, then remained constant for 1 min at 150 °C, increasing thereafter to 220 °C (230 °C for dodecyl ester) at a rate of 10 °C/min. A 1:20 split injection ratio was used with He as the carrier gas. Chromatographic peaks were recorded and integrated with Trace-ThermoQuest computer software. Before GC analysis, we added 0.5 mL of methyl palmitate in isooctane (10 mM) as an internal standard and 1 mL of aqueous saturated $NaHCO_3$ solution to 0.5 mL of each sample. These mixtures were incubated at room temperature for 24 h on an orbital shaker (200 rpm). The organic phase was separated by centrifugation at 3000 rpm.

RESULTS AND DISCUSSION

Isolation of Endophytic Fungi. In this work, we isolated a total of 94 fungi from plant samples. **Table 1** shows the number of strains isolated from each plant species and gives the corresponding identification codes.

Preliminary Screening. We screened all of the microbial strains isolated for hydrolytic activity in plates containing solid medium supplemented with tributyrin as a substrate. In this test, activity is detected as the formation of a clear halo around the colony (*14*). The use of tributyrin as a substrate for the detection of microbial lipolysis has been called into question by some authors (*15*) because, in addition to most lipases, some esterases also hydrolyze this substrate. However, tributyrin is a good substrate for a rapid screening test because it disperses in water more easily than true lipase substrates such as triolein (*16*). A total of 76 microorganisms, corresponding to 80% of the investigated strains, generated a clear halo (**Table 2**). Halos of this type are observed only if the fatty acids released are at least partially soluble in water. The observed clarification of the medium results from a decrease in the size of the hydrolyzed emulsified particles, which in turn causes a decrease in light diffusion (*17*).

Secondary Screening. We assessed mycelium-bound lipase activity by monitoring the esterification of an alcohol with a carboxylic acid in an organic solvent (*14*). We assessed the ability of all 76 strains selected in the first screening to act as biocatalysts for the synthesis of propyl oleate. **Figure 1** shows the diameter of the halo formed in tributirin assays plotted against lipase activity as evaluated on the basis of propyl oleate concentration. Ten of the tested strains (8, 9A, 10A, 10E, 16C, 18A, 23A, 24A, 24C, and 27F) gave significant ester yields. However, comparison of the results of preliminary and secondary screening showed an absence of general correlation between hydrolytic and synthetic activities, as reported in other studies (*3*, *18*). Thus, the lipase from strain 16C, which displayed low levels of hydrolytic activity, was very active in the synthesis of propyl oleate. In contrast, lipases from strains 10E, 18A, 23A, 24A, and 24C, which were highly active against tributyrin, displayed only moderate levels of activity in propyl oleate synthesis. Nevertheless, strain 10A clearly gave the highest hydrolytic and synthetic activities of any of the tested strains. The differences between levels of hydrolytic and synthetic activity may be accentuated by the fact that the tributirin assay measures both extracellular and mycelium-bound hydrolase activity on a hydrophilic medium whereas propyl oleate synthesis indicates the presence of mycelium-bound lipase activity on a hydrophobic medium.

As shown in **Figure 1**, strain 16C presented considerable cell-bound catalytic activity. However, this strain proved to be very difficult to maintain on agar after repeated platings. We therefore used only strain 10A for subsequent experiments. Strain 10A, isolated from *Foeniculum vulgare* (fennel), was identified in our laboratory as *Rhizopus oryzae*, an identification confirmed by the CBS (Central Bureau voor Schimmelcultures, Baarn, Holland).

Improvement of the Mycelium-Bound Lipase Activity of the Selected Strain (*R. oryzae*). *Effect of Water Content on Lipase Activity.* Water activity (a_w) as a measure of water content is one of the most important factors, other than the nature of the solvent, affecting enzyme activity in organic solvents. Once the nature of the biocatalyst and the nature of the solvent are fixed, the water sorption isotherm depends on the amount of added water (*13*). A minimum amount of water, depending on the type of organic solvent used and the characteristics of the immobilization support, is known to be strictly required for the solvation of enzymes. However, the presence of excess water favors hydrolysis over synthesis reactions (*19*). Water content may therefore affect reaction rate, yield, and the stability of enzymes (*20*). Thus, water content must generally be strictly controlled for ester synthesis (*21*). Moreover, the water content requirements for biocatalysis are highly dependent on the solvent (*22*), with highly polar solvents tending to strip essential water from the enzyme (*23*). The mycelium-bound lipase of *R. oryzae* was most efficient at the esterification of linoleic acid with 1-propanol if used dried or after adding small amounts of water (5 μL) (**Figure 2**). The addition of 10 or 20 μL of water led to a clear decrease in ester formation after 5 min of reaction. The addition of 5 μL of water to the reaction system corresponds to the same amount of mycelium used. Considering the low solubility of the water in isooctane, it is possible to assume that biocatalyst acts as a sorbent of the added water without any great influence on its activity, determined considering the initial esterification rate. Addition of 7 μL of water does not cause any considerable loss of activity either. However, when 10 μL or more water was added, enzymatic activity showed a clear decrease. Although we had not measure the water activity of

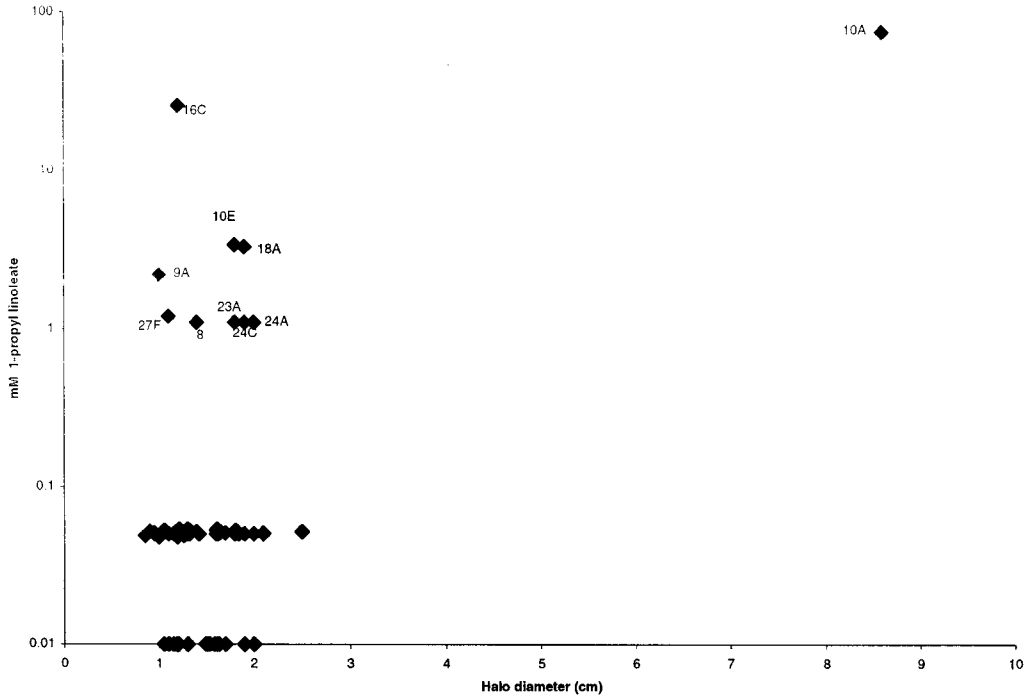

Figure 1. Semilogarithmic plot of hydrolytic activity vs synthetic activity of the isolated strains. Hydrolytic activity was measured as the diameter of the cleared zone after 5 days of incubation at 25 °C. Synthetic activity was measured as the concentration of 1-propyl oleate obtained following the incubation of the lyophilized mycelium in a solution of oleic acid and 1-propanol in isooctane for 3 h at 26 °C.

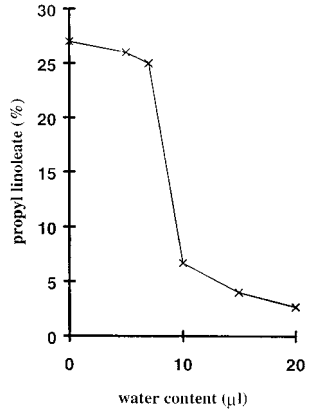

Figure 2. Effect of water content on the esterification of linoleic acid with 1-propanol catalyzed by *R. oryzae* resting cells. Reactions were carried out in isooctane at 26 °C for 5 min.

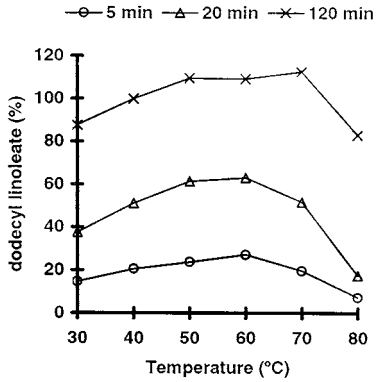

Figure 3. Effect of temperature on the esterification of linoleic acid with 1-dodecanol catalyzed by *R. oryzae* resting cells. Reactions were carried out in isooctane for 5 min, 20 min, and 2 h.

the system, it is well-known that an increase of the contents of water causes an increase of the a_w of the system (*13*). These results are similar to the ones found by Koller et al. (*24*). These authors, using extracellular enzymes isolated from *R. oryzae* and *Rhizopus niveus* and adsorbed onto a microporous polypropylene support, showed that lipases displayed optimal activity at very low a_w levels. However, the amount of water required for high levels of catalytic activity differs greatly between lipases. Some, such as the enzymes from *Rhizomucor miehei*, *Candida antarctica*, and *Pseudomonas glumae*, retain high levels of activity even after drying over a molecular sieve. Other lipases, such as those from *Candida rugosa* and *Candida deformans*, seem to require higher levels of water for high levels of activity (*21*, *22*).

Effect of Temperature on Lipase Activity. We carried out dodecyl linoleate synthesis reactions to study the effect of temperature on the mycelium-bound lipase activity of *R. oryzae* (**Figure 3**). The optimal temperature for esterification was 60 °C, with over 20% transformation in 5 min, 60% transformation in 20 min, and 100% conversion after 2 h. The enzyme transformed 100% of the linoleic acid after 2 h of reaction, whether the reaction was performed at 50 or 70 °C. Above 80 °C, this membrane-bound lipase retained about 80% activity, giving yields similar to those obtained at 30 °C. Yields of dodecyl linoleate greater than 100% were obtained in some cases, indicating the presence of residual linoleic acid originating from the sunflower oil component of the growth medium (*12*).

This mycelium-bound lipase was stable at 60 and 70 °C (**Figure 4**). Increases in preincubation time (30 min to 2.5 h) and preincubation temperature (30 to 60 or 70 °C) slightly increased synthetic activity. Ester yields of 87.6, 96.5, and 93%, respectively, were obtained after 2 h of reaction at 30 °C depending on the preincubation temperature used.

In a similar study, Razak et al. (*25*) found that the optimal temperature for activity of the membrane-bound lipase obtained from a *R. oryzae* strain was 37 °C. At 50 °C, it retained more than 90% of its initial activity after 30 min, but at 70 °C, only 10% of the initial activity was observed. Our mycelium-bound

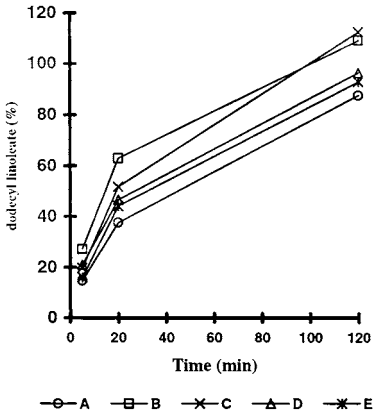

Figure 4. Temperature stability of the membrane-bound lipase. The enzyme was incubated at 30 or 150 min at various temperatures, cooled, and assayed at various temperatures and reaction times. (**A**) Preincubation for 30 min at 30 °C, reaction at 30 °C. (**B**) Preincubation for 30 min at 60 °C, reaction at 60 °C. (**C**) Preincubation for 30 min at 70 °C, reaction at 70 °C. (**D**) Preincubation for 150 min at 60 °C, reaction at 30 °C. (**E**) Preincubation for 150 min at 70 °C, reaction at 30 °C.

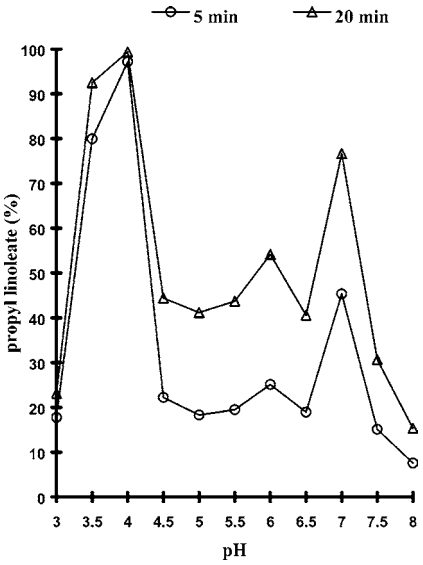

Figure 5. Effect of pH on the esterification of linoleic acid with 1-propanol catalyzed by *R. oryzae* resting cells. Reactions were carried out in isooctane at 26 °C for 5 and 20 min.

lipase therefore functions at higher temperatures and is more heat stable than that described by Razak et al. (*25*).

Effect of pH on Lipase Activity. **Figure 5** shows the variation in activity observed between pH 3 and pH 8. Two peaks in activity were observed, at pH 4 and pH 7. This broad activity range differs from that reported for most fungal lipases, which are active only in acidic conditions. Such lipases include those from *Rhizopus rhizodiformis* (pH 6.0) (*26*), *Rhizopus delemar* (pH 5.6) (*27*), and *R. oryzae* (pH 6.0) (*25*). Namboodiri and Chattopadhyaya (*28*) found that the optimal pH for the *Aspergillus niger* lipase was between pH 5.0 and pH 6.0 in phosphate buffer, although the enzyme was active from pH 5.0 to pH 7.5; activity was gradually lost below pH 5.0 and above pH 7.5. The mycelium-bound lipase described here was active in both acidic and neutral conditions.

Endophytic fungi are clearly a potentially useful source of mycelium-bound lipases. The mycelium-bound lipase from the strain of *R. oryzae* isolated from *F. vulgare* requires very little water for catalytic activity, and the synthesis of propyl linoleate catalyzed by the *R. oryzae* mycelium was optimal at pH 3.5−4 and pH 6−7. Resting cells of *R. oryzae* displayed marked heat stability because activity began to decrease markedly only at temperatures above 80 °C.

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