Mycoscience (2005) 46:119–126
DOI 10.1007/s10267-004-0221-9

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Extracellular enzymatic activity profiles in fungi isolated from oil-rich environments

Received: June 23, 2004 / Accepted: November 27, 2004

Abstract About 34 wild fungal species associated with edible oil mill wastes were isolated by the serial dilution technique. Methods for rapid screening of fungal species against production of extracellular enzymes such as amylase, protease, cellulase, and lipase are reported. Among all the species, *Aspergillus versicolor* exhibited high amlylolytic and gelatinolytic activity, whereas *Penicillium citrinum* showed only high amylolytic activity. Maximum cellulolytic activity was recorded for *Absidia corymbifera, As. niger, Cunninghamella echinulata, Curvularia lunata, Fusarium solani, Mucor racemosus, Paecilomyces variotii*, and *Syncephalastrum racemosum*. The fungal species *Ab. corymbifera, As. fumigatus, As. japonicus, As. nidulans, As. terreus, Cun. verticillata, Cur. pallescens, F. oxysporum, Geotrichum candidum, M. racemosus, Pe. citrinum, Pe. frequentans, Rhizopus stolonifer*, and *Trichoderma viride* exhibited maximum lipase activity. This study confirms the isolated fungi present on a wide range of substrates in the ambient environment, and these results could provide basic data for further investigations on fungal extracellular enzymes.

Key words Amylase · Cellulase · Lipase · Oil mill waste · Protease

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Introduction

Fungi, being ubiquitous in distribution, are highly successful in survival because of their great plasticity and physiological versatility. Fungi thrive well in inhospitable habitats with environmental extremes because of their efficient enzyme system. Among the varied mechanisms for adaptability of fungi to environmental extremes and for the utilization of their trophic niche, their ability to produce extracellular enzymes are of great survival value. Fungi are involved in the biodegradation of undesirable materials or compounds and convert them into harmless, tolerable, or useful products. The undesirable materials include sewage waste from domestic and industrial complexes, plant, animal, and agricultural wastes, pesticides, oil spills, and detergents. The role of fungi in bioremediation processes in various environments has been well documented. Besides waste disposal, bioconversion by fungal activity results in the production of a vast number of useful substances.

Naturally, all the fungi produce a large number of enzymes to break down the complex materials for their growth. Knowledge of the enzymatic activities to be used for biotechnological purposes, therefore, has to be the basis of all studies. For this purpose, different methods for detection and determination of enzyme activity of oil mill waste-based fungi have been warranted. Although recent surveys have underlined the potential of unconventional and extreme environments as a source of natural biodiversity for the isolation and selection of useful microorganisms (Bull et al. 1992), to our knowledge only a few studies have been carried out on mycoflora from edible oil wastes. Due to usage of vegetable oils for cooking, these oils are released into the open environment both at the production point and by domestic users. To keep the environment clean, these oils should be degraded by using environment-friendly technology. Thus, we launched this study and report here the results of a large-scale screening survey on the production of extracellular enzymes by fungi isolated from habitats of oil-polluted environments.

Materials and methods

Oil mill wastes were collected from five different stations of Tamil Nadu, India, and fungi were isolated by serial dilution technique (Akano and Atanda 1990) and identified (Gopinath 1998). The isolated fungi were maintained on potato dextrose agar slants. The use of solid media for the detection of various enzymes, first suggested by Hankin and Anagnostakis (1975), was referred to as a basic method in the present investigation. The fungi were screened for their ability to produce extracellular amylase, protease, cellulase, and lipase on solid media. The relative enzyme activity was estimated by measuring the formation of a clear zone and growth by the radial limit.

Amylolytic enzyme assay

Nutrient agar [NaCl 5.0 g, peptone 5.0 g, beef extract 5.0 g, glucose 10.0 g, agar 20.0 g/l (obtained from Difco] and 0.2% soluble starch (Sigma) in distilled water were autoclaved separately, mixed, and cooled (pH 6). About 20 ml of media was poured into each sterile Petri plate and allowed to solidify. The fungal cultures were inoculated at the center of the plate containing sterile medium, using a pinpoint inoculum, and incubated at room temperature for a period of 7 days. After the incubation period, the plates were flooded with iodine solution. Formation of a yellow zone around the fungal colony and the development of blue color on the surface excluding the area around colonies indicated the hydrolysis of starch (Gopinath et al. 2003a).

Gelatinolytic enzyme assay

Nutrient agar medium in distilled water was adjusted to pH 6.0. An 8% solution of gelatin (Sigma) in water was sterilized separately and was added to the nutrient agar at a rate of 5 ml per 100 ml nutrient medium. The gelatin agar plates were inoculated with a pinpoint inoculum in the center and incubated at room temperature. After incubation, complete degradation of the gelatin was seen, as a clear zone around colonies, in the somewhat opaque agar. When the plate was flooded with an aqueous saturated solution of ammonium sulfate, a precipitate was formed that made the agar more opaque and enhanced the clear zones around the colonies.

Cellulolytic enzyme assay

Czapek dox agar medium (sucrose 30.0 g, sodium nitrate 3.0 g, magnesium sulfate 0.5 g, potassium chloride 0.5 g, iron(III) sulfate 0.01 g, dipotassium hydrogen phosphate 1.0 g, agar-agar 13.0 g/l, pH 7.0) with 1% w/v cellulose (Sigma) in distilled water was autoclaved and cooled. About 20 ml of media was poured into a sterile Petri dish and allowed to sodify. The fungal cultures were inoculated at the center of the plate using a pinpoint inoculum and incubated at room temperature for a period of 7 days. Colonies that produced a clear zone on cellulose agar plates were measured (Tanaka et al. 1980).

Lipolytic enzyme assays

Lipase activity on Tween-20 agar plates

The culture medium was prepared by adding peptone 10.0 g, NaCl 5.0 g, $CaCl_2 \cdot 2H_2O$ 0.1 g, and agar 20.0 g in 1000 ml water and autoclaved for 20 min; 10 ml Tween-20 (Sigma) was separately sterilized and added into the autoclaved medium, and the pH was adjusted to 6.0. About 20 ml the medium was poured into each Petri dish and inoculated at the center using a pinpoint inoculum of the test fungus. Lipolytic activity was indicated by the appearance of a visible precipitate, resulting from the deposition of crystals of the calcium salt formed by the fatty acid liberated by the enzyme, or as a clearing of such a precipitate around a colony due to complete degradation of the salt of the fatty acid. At regular intervals of 24-h incubation, each plate was examined and measurements were taken to monitor lipolytic activity (Sierra 1957).

Lipase activity on LBT agar plates

The medium was prepared by adding tryptone 10.0 g, NaCl 5.0, yeast extract 5.0 g, distilled water 11 (pH 7.0), 0.5% tributyrin (Sigma), 1.5% agar, and homogenizing in a Waring blender at setting 35 for 5 min. The solution was then autoclaved and dispensed into sterile Petri dishes. The plates were inoculated and incubated at room temperature for a period of 7 days. The development of a clear zone is an indication of lipolytic activity, and its area is a measure of the extent of activity (Lee and Rhee 1993). At regular intervals of 24-h incubation, each plate was examined and measurements on the area of the cleared zone were taken to monitor lipolytic activity.

Tributyrin method

Modified lipase medium was prepared by adding 10.0 g peptone and 1.0 g yeast extract to 1 l 0.1 M phosphate buffer, pH 7.0, and then autoclaving (Roberts et al. 1987). Then, 1% sterilized glucose was added to the sterile peptone–yeast extract solution. Sterile Erlenmeyer flasks (250 ml) containing 100 ml sterile yeast-extract-peptone medium with glucose were inoculated with 2 ml (1×10^7 spores/ml) of spore suspension of 7-day-old culture of the test fungi and incubated with incidental illumination at 30°C and 110 rpm in a rotary shaker. After 5 days the cultures were filtered through a filter paper to remove the mycelium and then passed through sterile 0.22-μm filters. The sterile culture filtrates were stored at −20°C until further use (Roberts et al. 1987; Gopinath et al. 2003b). The culture filtrate is the source of enzyme and is used to study the extracellular lipolytic activity on tributyrin agar plates as described next.

Tributyrin homogenate was prepared by adding 1 ml tributyrin to 99 ml 0.05 M phosphate buffer, pH 7.0, and homogenized in a Waring blender at setting 35 for 5 min (Lawrence et al. 1967). The agar medium (1.3%) was prepared by adding 3.9 g agar to 270 ml 0.05 M phosphate buffer, pH 7.0. The mixture was heated until a homogenate agar solution was obtained; 30 ml tributyrin homogenate was then added to the molten agar with stirring. The solution was then autoclaved and dispensed into sterile Petri dishes. Wells of 10 mm diameter were made in the agar plate with a sterile cork borer and the culture filtrate (crude enzyme) was loaded into each well. One of the wells received only the autoclaved filtrate, which formed the control. The plates were incubated at 30°C and observed for lipolytic activity over a period of 72 h. The clear zone of tributyrin homogenate around the well indicated the lipolytic activity present in the culture filtrate.

Spectrophotometric assay for lipase activity

Lipase activity was assayed quantitatively by using 4-nitrophenyl palmitate as the substrate (Winkler and Stuckmann 1979; Gopinath et al. 2002, 2003b). First, 10 ml isopropanol containing 30 mg 4-nitrophenyl palmitate (Sigma) was mixed with 90 ml 0.05 M sodium phosphate buffer (pH 8.0) containing 207 mg sodium deoxycholate and 100 mg gum arabic. A total amount of 2.4 ml freshly prepared substrate solution was prewarmed at 37°C and mixed with 0.1 ml enzyme solution (as prepared in the tributyrin method). After 15 min incubation at 37°C, absorbance at 410 nm was measured against an enzyme-free control. One enzyme unit was defined as 1 μmol 4-nitrophenol enzymatically released from the substrate in milliliters per minute (ml/min).

Results and discussion

Several reviewers (Bull et al. 1992; Steele and Stowers 1991) still stress the fact that, although advances in genetics and microbial physiology are having a strong impact on enzyme production, screening programs for the selection of microorganisms able to produce bioactive molecules continue to be an important aspect of biotechnology. Therefore, new commercial opportunities could be revealed by systematic programs of screening microbial strains aimed at well-defined industrial targets (Buzzini and Martini 2002). In the present assessment, about 34 wild fungal species associated with edible oil mill wastes were isolated by serial dilution technique from samples obtained at five different stations in Tamil Nadu, India. Data on the distribution of recovered fungal strains are given in Table 1.

A total of 1524 colonies of wild fungal strains were isolated and classified into 34 species belonging to 17 genera. Among isolated fungi, *Aspergillus (As.) niger* was most commonly observed, followed by the following fungal species described in decreasing rank: *As. flavus* > *Rhizopus stolonifer* > *As. terreus* > *As. flavipes* > *As. versicolor* > *As. fumigatus*, explaining their ubiquitous nature. Some species were appeared specific to a particular station, and they are probably sensitive to the particular environment. From a comparative analysis, it may be seen that the maximum number of colonies was recorded at station III (455 colonies), which probably resulted from extraneous factors such as an open drainage system, increased levels of dumping of garbage, and a dirty narrow road that favor the growth of a large population of these fungi. The isolated fungal species from different stations were examined for their ability to produce extracellular enzymes.

Amylolytic activity

Assays on solid media were made semiquantitatively by measuring the diameter of the zones formed in the agar and relating this zone size to the colony size. In this manner, the enzymatic activities of fungal species were determined. In the assay method for amylolytic activity, the formation of a yellow zone around a fungal colony indicated the hydrolysis of starch. The symbols + (low activity), ++ (moderate activity), and +++ (high activity) indicate a diameter <4 cm, 4–7 cm, and >7 cm, respectively (Table 2). Among the species tested, most could not hydrolyze starch even after 7 days of incubation. These species include *Absidia (Ab) corymbifera, Acremonium strictum, As. fumigatus, As. japonicus, As. terreus, Cladosporium (Cl.) cladosporioides, Cunninghamella (Cun.) echinulata, Cun. verticillata, Curvularia (Cur.) lunata, Fusarium (F.) solani, Fusarium* sp., *Geotrichum (G.) candidum, Mucor (M.) racemosus, Paecilomyces (Pa.) variotii, Penicillium (Pe.) frequentans, Pe. funiculosum, Pe. oxalicum, Rhizomucor (R.) miehei, Rhizopus stolonifer, Scopulariopsis (Sc.) brevicaulis, Syncephalastrum (Sy.) racemosum*, and *Trichoderma (T.) viride*. All other species exhibited low amylolytic activity, except *As. flavus, As. versicolor*, and *Pe. citrinum.* Among these species, *As. versicolor* and *Pe. citrinum* showed high activity whereas *As. flavus* exhibited moderate activity.

Hankin and Anagnostakis (1975) first used solid media to test the enzyme activities of seven plant pathogenic and six saprophytic fungi, as well as a sample of leaf compost. Moscoso and Rosato (1987) analyzed extracellular amylase, lipase, and protease produced by haploids, diploids, and heterocaryons of *As. nidulans*. Three morphologically normal strains and 8 morphological mutants as well as various genetic combinations of the 11 strains were examined in solid culture medium containing specific substrate. For amylase and protease, the highest values were reached by some diploid and heterocaryons and for lipase by 1 morphological strain. In our earlier report, using this assay system we made a detailed study of amylase production by *As. versicolor* (Gopinath et al. 2003a).

Gelatinolytic activity

The production of proteases has so far been studied mainly for their implications in the beer and wine industry

Table 1. Frequency occurrence of mycoflora isolated from five stations of Tamil Nadu, India

Fungal strain	Station I		Station II		Station III		Station IV		Station V		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Absidia corymbifera	0	0	4	2.6	9	2.0	13	3.1	13	3.9	39	2.6
Acremonium strictum	3	1.8	1	0.7	6	1.3	0	0	0	0	10	0.7
Aspergillus clavatus	8	4.8	5	3.3	13	2.9	15	3.6	14	4.2	55	3.6
As. flavipes	7	4.2	7	4.6	24	5.3	18	4.3	20	6.0	76	5.0
As. flavus	13	7.7	11	7.3	50	11.0	23	5.5	23	6.9	120	7.9
As. fumigatus	8	4.8	4	2.7	24	5.3	22	5.3	15	4.5	73	4.8
As. japonicus	7	4.2	5	3.3	19	4.2	20	4.8	13	3.9	64	4.2
As. nidulans	4	2.4	10	6.6	24	5.3	15	3.6	13	3.9	66	4.3
As. niger	11	6.5	11	7.3	50	11.0	61	14.7	37	11	170	11.2
As. ochraceus	8	4.8	7	4.6	12	2.6	18	4.3	14	4.2	59	3.9
As. terreus	10	6.0	7	4.6	24	5.3	27	6.5	18	5.4	86	5.6
As. versicolor	9	5.4	5	3.3	24	5.3	23	5.5	14	4.2	75	4.9
Cladosporium cladosporioides	2	1.2	5	3.3	6	1.3	7	1.7	11	3.3	31	2
Cunninghamella echinulata	0	0	0	0	9	2.0	3	0.7	0	0	12	0.8
Cun. verticillata	0	0	4	2.7	5	1.1	6	1.4	4	1.2	19	1.2
Curvularia lunata	3	1.8	2	1.3	5	1.1	7	1.7	6	1.8	23	1.5
Cur. pallescens	7	4.2	5	3.3	14	3.1	7	1.7	9	2.7	42	2.8
Fusarium oxysporum	4	2.4	2	1.3	12	2.6	2	0.5	1	0.3	21	1.4
F. solani	1	0.6	2	1.3	0	0	1	0.2	1	0.3	5	0.3
Fusarium sp.	5	3.0	3	2.0	6	1.3	2	0.5	7	2.1	23	1.5
Geotrichum candidum	2	1.2	2	1.3	4	0.9	4	1.0	3	0.9	15	1
Mucor mucedo	8	4.8	4	2.7	10	2.2	1	0.2	6	1.8	29	1.9
M. racemosus	4	2.4	4	2.7	6	1.3	6	1.4	7	2.1	27	1.7
Paecilomyces variotii	0	0	0	0	9	2.0	0	0	14	4.2	23	1.5
Penicillium citrinum	8	4.8	10	6.6	12	2.6	13	3.1	14	4.2	57	3.7
Pe. frequentans	3	1.8	2	1.3	0	0	3	0.7	0	0	8	0.5
Pe. funiculosum	5	3.0	3	2.0	20	4.4	23	5.1	16	4.8	67	4.4
Pe. oxalicum	4	2.4	2	1.3	12	2.6	14	3.4	11	3.3	43	2.9
Rhizomucor miehei	1	0.6	2	1.3	0	0	3	0.7	1	0.3	7	0.5
Rhizopus stolonifer	12	7.1	9	6.0	28	6.2	33	8.0	14	4.2	96	6.3
Scopulariosis brevicaulis	4	2.4	2	1.3	0	0	8	1.9	0	0	14	0.9
Syncephalastrum racemosum	1	0.6	3	2.0	6	1.3	9	2.2	4	1.2	23	1.5
Trichoderma viride	3	1.8	4	2.7	11	2.4	6	1.4	11	3.3	35	2.3
Trichothecium roseum	3	1.8	4	2.7	1	0.2	3	0.7	0	0	11	0.7
Total number of species	168		151		455		416		334		1524	

(Bilinkski and Stewart 1990; Dizy and Bisson 2000; Strauss et al. 2001). To determine the gelatinolytic activity, the fungi were inoculated in the center of the Petri plate containing nutrient agar with 0.4% gelatin. The symbols +, ++, and +++ were used as already indicated. Most of the fungi tested exhibited gelatinolytic activity by forming a clear zone around fungal colonies when the plate was flooded with an aqueous saturated solution of ammonium sulfate. Among these, *As. versicolor* exhibited maximum gelatinolytic activity, whereas moderate activity was recorded in *As. niger, Cun. verticillata, F. oxysporum, R. miehei*, and *Sy. racemosum*. Low activity was registered by *Ac. strictum, As. clavatus, As. flavipes, As. flavus, As. fumigatus, As. nidulans, As. terreus, Cun. echinulata, Cur. lunata, Fusarium* sp., *G. candidum, M. racemosus, Pa. variotii, Pe. funiculosum, Pe. oxalicum, T. roseum*, and *T. viride.* Furthermore, species such as *Ab. corymbifera, As. japonicus, As. ochraceus, Cl. cladosporioides, Cur. pallescens, F. solani, M. mucedo, Pe. citrinum, Pe. frequentans, R. stolonifer*, and *Sc. brevicaulis* showed no gelatinolytic activity (see Table 2). Previously, gelatin liquefaction was detected in less than 50% of the cultures, and only 7 strains showed strong activity of 198 strains screened (Federici 1982). Papini and Mancianti (1996) have failed to find trypsin and chymotrypsin activities when screening *Microsporum canis* isolates. Some researchers used casein-amended agar plates to screen the yeast strains (Buzzini and Martini 2002).

Cellulolytic activity

To determine the cellulolytic activity, the fungi were inoculated into Czapek-dox agar medium with 1% cellulose. Among the fungi tested, most of them showed cellulolytic activity. However, *As. ochraceus, Cl. cladosporioides, Cun. verticillata, F. oxysporum*, *M. mucedo*, and *R. stolonifer* could not hydrolyze cellulose even after 7 days of incubation. Maximum cellulolytic activity was recorded for *Ab. corymbifera, As. niger, Cun. echinulata, Cur. lunata, F. solani, M. racemosus, Pa. variotii*, and *Sy. racemosum*. Low cellulolytic activity was exhibited by *Ac. strictum, As. flavipes, As. fumigatus, Cur. pallescens, Fusarium* sp., *Pe. citrinum, Pe. frequentans, Pe. oxalicum, R. miehei, Sc. brevicaulis*, and *T. viride.* All other fungi showed moderate activity (Table 2). A similar substrate has been

Table 2. Screening fungi for extracellular enzymes

Fungal strain	Amylolytic activity	Gelatinolytic activity	Cellulolytic activity
Absidia corymbifera	ND	ND	+++
Acremonium strictum	ND	+	+
Aspergillus clavatus	+	+	++
As. flavipes	+	+	+
As. flavus	++	+	++
As. fumigatus	ND	+	+
As. japonicus	ND	ND	++
As. nidulans	+	+	++
As. niger	+	++	+++
As. ochraceus	+	ND	ND
As. terreus	ND	+	++
As. versicolor	+++	+++	++
Cladosporium cladosporioides	ND	ND	ND
Cunninghamella echinulata	ND	+	+++
Cun. verticillata	ND	++	ND
Curvularia lunata	ND	+	+++
Cur. pallescens	+	ND	+
Fusarium oxysporum	+	++	ND
F. solani	ND	ND	+++
Fusarium sp.	ND	+	+
Geotrichum candidum	ND	+	++
Mucor mucedo	+	ND	ND
M. racemosus	ND	+	+++
Paecilomyces variotii	ND	+	+++
Penicillium citrinum	+++	ND	+
Pe. frequentans	ND	ND	+
Pe. funiculosum	ND	+	++
Pe. oxalicum	ND	+	+
Rhizomucor miehei	ND	++	+
Rhizopus stolonifer	ND	ND	ND
Scopulariosis brevicaulis	ND	ND	+
Syncephalastrum racemosum	ND	++	+++
Trichoderma viride	ND	+	+
Trichothecium roseum	+	+	++

+++, high activity; ++, moderate activity; +, low activity; ND, not detected

used to screen yeast strains from tropical environments. *Aureobasidium pullulans* was assayed for cellulolytic activity because it can utilize cellobiose, the main end product of cellulose hydrolysis, as the sole carbon source (Dennis and Buhagiar 1973). However, Federici (1982) explained that none of the isolates of *A. pullulans* was able to degrade either cellulose or carboxymethyl cellulose. A cellulolytic fungus, *Humicola fuscoatra*, isolated from paper mill effluent-irrigated soil, was identified by plate assay using cellulose as substrate (Rajendran et al. 1994).

Lipolytic activity

Lipases are gaining industrial interest with applications in laundry detergents and in dairy industries (Burden and Eveleight 1990). Because the fungi were harbored in oil-rich environments, we paid much attention to screening the lipolytic enzymes, and different methods for detection and determination of lipase activity of oil mill waste fungi were envisioned. For detecting lipolytic fungi of higher potential, agar plates with Tween-20, tributyrin, and solutions with 4-nitrophenylpalmitate were used as the substrates.

Data on the extracellular lipase activity of isolated fungi on Tween-20 are given in Table 3. The development of a clear crystal zone of Tween-20 around the fungus was an indication of lipolytic activity, and this zone was measured. Extracellular lipase activity of all the fungi except a few was detected by the hydrolysis of Tween-20 from the first day of incubation. The fungi *Ac. strictum, As. fumigatus, As. nidulans, G. candidum, Pa. variotii, Pe. funiculosum, Pe. oxalicum, Sy. racemosum, T. roseum*, and *T. viride* could hydrolyze Tween-20 from the first day of incubation. All other fungi exhibited a zone of hydrolysis of Tween-20 from 48 h incubation. However, a few species, *As. niger, As. versicolor, Pe. citrinum*, and *Pe. frequentans* colonies, showed lipolytic activity from 72 h incubation. The peak activity (>7 cm) occurred at 168 h in *As. fumigatus* and *As. nidulans*, and at 144 h in *M. racemosus*. Weak lipolytic activity (<4 cm) was registered in *As. clavatus, As. flavus, As. ochraceus, As. versicolor, Fusarium* sp., *G. candidum, M. mucedo, Pa. variotii, Pe. frequentans, Pe. oxalicum*, and *Sy. recemosum*. Interestingly, the species *Cun. echinulata, Cun. verticillata, F. oxysporum, F. solani*, and *Rhizopus stolonifer* could not hydrolyze Tween-20 even after 7 days incubation, indicating that they are lipase-negative species. All other species registered medium activity (4–7 cm). Further, with an increase in the period of incubation there was an increase in extracellular lipolytic activity, with a concomitant increase in mycelial growth. It was suggested that the pri-

Table 3. Screening fungi for extracellular lipolytic enzyme

Fungal strain	Tween-20	Luria-Bertani Tributyrin (LBT)	Tributyrin
Absidia corymbifera	++	++	++
Acremonium strictum	++	+	++
Aspergillus clavatus	+	+	++
As. flavipes	++	+	++
As. flavus	+	+	++
As. fumigatus	+++	+	++
As. japonicus	++	+	++
As. nidulans	+++	+	++
As. niger	++	+	++
As. ochraceus	+	+	++
As. terreus	++	+	+++
As. versicolor	+	+	++
Cladosporium cladosporioides	++	+	++
Cunninghamella echinulata	ND	++	++
Cun. verticillata	ND	+++	++
Curvularia lunata	++	ND	++
Cur. pallescens	++	+	++
Fusarium oxysporum	ND	+++	+++
F. solani	ND	ND	ND
Fusarium sp.	+	++	++
Geotrichum candidum	+	+	+++
Mucor mucedo	+	+	+
M. racemosus	+++	+++	++
Paecilomyces variotii	+	++	++
Penicillium citrinum	++	+	+++
Pe. frequentans	+	++	+++
Pe. funiculosum	++	++	++
Pe. oxalicum	+	+	++
Rhizomucor miehei	++	++	++
Rhizopus stolonifer	ND	+++	++
Scopulariosis brevicaulis	++	+	++
Syncephalastrum racemosum	+	+++	+
Trichoderma viride	++	+++	+++
Trichothecium roseum	++	+	++

+++, high activity; ++, moderate activity; +, low activity; ND, not detected

mary role of calcium ion was to remove fatty acids formed in the hydrolysis as insoluble calcium soaps, and thus change the interfacial substrate–water relationship to a favorable condition for enzyme action.

Extracellular fungal lipase activity was further detected using LBT agar plates containing 0.5% tributyrin. The clear zone around the fungal colony showed that these fungal strains produced extracellular lipase. It is evident from Table 3 that all the species, except *Cur. lunata* and *F. solani*, exhibited lipolytic activity. These 2 species could not hydrolyze tributyrin even after 168 h incubation, indicating that these 2 are lipase-negative species using this substrate. Of the 34 species, some, namely, *As. clavatus, As. flavus, As. nidulans, As. ochraceus, As. terreus, Cur. pallescens, G. candidum*, and *Pe. funiculosum*, exhibited lipolytic activity from 48 h incubation, whereas *Ac. strictum, As. versicolor, M. mucedo*, and *T. roseum* showed lipolytic activity from 72 h incubation. Species such as *As. japonicus* and *Sc. brevicaulis* showed activity from 96 h incubation, and the species *As. fumigatus* exhibited lipolytic activity from 120 h incubation. All other fungi exhibited lipolytic activity from 24 h incubation. The peak activity (>7 cm) was recorded at 96 h by *F. oxysporum* and *M. racemosus*, 72 h by *Rhizopus stolonifer*, and 168 h by *Cun. verticillata, Sy. racemosum*, and *T. viride*. The weakly lipolytic (<4 cm) fungi are *Ac. strictum,* all *Aspergillus* spp., *Cl. cladosporioides, Cur. pallescens, G. candidum, M. mucedo, Pe. citrinum, Pe. oxalicum, Sc. brevicaulis*, and *T. roseum.* Interestingly, in *Cl. cladosporioides* there was an increase in the extracellular lipase activity concomitant with the increase in the period of incubation without a corresponding increase in the mycelial growth.

The isolated fungi were also assayed for lipase activity by the tributyrin agar diffusion method. Tributyrin is a convenient substrate, as it is easily dispersed in water by shaking or stirring without the addition of any emulsifiers. Further, tributyrin is a very strong surface-active substance, and its hydrolysis can be followed by measuring the increase in the diameter of the clear zone. The extracellular lipolytic activity of fungal species (by adding culture filtrate) is indicated by zones of clearing in tributyrin agar plates. Tributyrin was lysed by all the species except *F. soloni,* as a negative species for the hydrolysis of tributyrin. *Ab. corymbifera, As. japonicus*, *As. versicolor, Fusarium* sp., *M. mucedo*, *Pa. variotii*, and *Sy. racemosum* exhibited a clear zone around the well only after 48 h incubation. The extracellular lipase from *As. ochraceus* exhibited lipolytic activity only after 72 h incubation, whereas the lipase from all other fungi

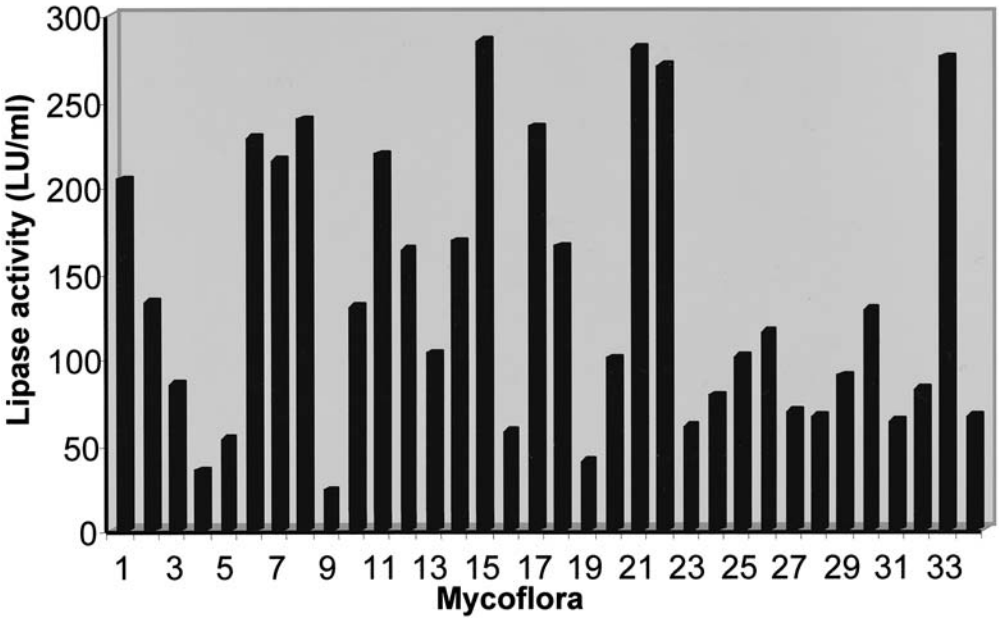

Fig. 1. Assay for lipase activity using 4-nitrophenyl palmitate. *1, Absidia corymbifera; 2, Acremonium strictum; 3, Aspergillus clavatus; 4, As. flavipes; 5, As. flavus; 6, As. fumigatus; 7, As. japonicus; 8, As. nidulans; 9, As. niger; 10, As. ochraceus; 11, As. terreus; 12, As. versicolor; 13, Cladosporium cladosporioides; 14, Cunninghamella echinulata; 15, Cun. verticillata; 16, Curvularia lunata; 17, Cur. pallescens; 18, Fusarium oxysporum; 19, F. solani; 20, Fusarium* sp.*; 21, Geotrichum candidum; 22, Mucor mucedo; 23, M. racemosus; 24, Paecilomyces variotii; 25, Penicillium citrinum; 26, Pe. frequentans; 27, Pe. funiculosum; 28, Pe. oxalicum; 29, Rhizomucor miehei; 30, Rhizopus stolonifer; 31, Scopulariosis brevicaulis; 32, Syncephalastrum racemosum; 33, Trichoderma viride; 34, Trichothecium roseum*

exhibited lipolytic activity from 24 h incubation. Maximum range of activity, i.e., above 5 cm, was registered in *As. terreus, F. oxysporum, G. candidum, Pe. citrinum, Pe. frequentans*, and *T. viride*. The fungi *M. mucedo* and *Sy. racemosum* are considered weakly lipolytic species (<2 cm) (see Table 3). Papini and Mancianti (1996) failed to find lipase activity when screening *Microsporum canis* isolates. In our previous studies, using this method as a preliminary trial, we succeeded in purifying the enzyme lipase from *G. candidum* (Gopinath et al. 2003b). Using tributyrin agar plates, several yeast strains from tropical environments were screened for lipase activity. Nevertheless, the observed zones of clearing could be the activity response of nonspecific esterases, which may have little or no activity against the long-chain triglycerides (Roberts et al. 1987). Hence, it is imperative to use another method to confirm lipase activity. However, recently tributyrin agar plates were used to investigate lipase production by new strains and found 18 strains to be positive (Cihangir and Sarikaya 2004).

Extracellular lipase activity of the isolated fungi was further confirmed and quantified by the spectrophotometric method formulated by Winkler and Stuckmann (1979) in which 4-nitrophenyl palmitate was used as a substrate (Fig. 1). Of the 34 species isolated from oil mill effluent, the following species exhibited maximum lipase activity (>200 units): *Ab. corymbifera, As. fumigatus, As. japnonicus, As. nidulans, As. terreus, Cun. verticillata, Cur. pallescens, G. candidum, M. mucedo*, and *Trichoderma viride.* Moderate activity (between 100 and 200 units) was registered by *Ac. strictum, As. ochraceus, As. versicolor, Cl. cladosporioides, Cun. echinulata, F. oxysporum, Fusarium* sp.*, Pe. citrinum*, *Pe. frequentans*, and *R. stolonifer*. The other fungal strains exhibited low activity.

From the foregoing investigations, it can be concluded that the substrate Tween-20 was not degraded by *Cun. echinulata, Cun. verticillata, F. oxysporum, F. solani*, and *R. stolonifer*. However, except *F. solani*, the other 4 species could degrade tributyrin. Lipase activity as detected and quantified using 4-nitrophenyl palmitate gave reliable and consistent results. From these results, lipolytic activity (high and moderate activity) was evidenced using Tween-20 and tributyrin methods by 18 and 31 species, respectively, whereas the spectrophotometric method supported 20 species. All our earlier studies mainly rely on tributyrin plates and assay with 4-nitrophenyl palmitate to detect lipolytic activity (Gopinath 1998; Gopinath et al. 2002, 2003a; Kumarevel et al. 2005).

The detection of enzymes in the culture media of the test fungi is an indication of the extracellularity of the enzyme, and the relative ease with which the enzymes were produced extracellularly probably explains the common occurrence of fungi in oil mill wastes. The above observations disclose new and interesting perspectives, demonstrating that fungi isolated from oil-rich environments actually represent a source of several enzymes potentially exploitable for biotechnological purposes. The production of various extracellular enzymes by the fungi harbored on oil mill wastes confirmed the saprophytic survival of these fungi in the ambient environment. A knowledge of the activities of

these fungi as shown in this study could go a long way to effectively containing them and hence preventing the deterioration of oil products and the environmental problems posed to the public. These methods may be useful when a large number of strains must be tested for extracellular enzyme activity or when conventional tests fail for assaying enzymatic induction in vitro.

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