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Extracellular Alkaline Lipase from a Novel Fungus *Curvularia* sp. DHE 5: Optimisation of Physicochemical Parameters, Partial Purification and Characterisation

Dina Helmy El-Ghonemy[1]*, *Mamdouh S. El-Gamal*[2], *Amir Elsayed Tantawy*[1] and *Thanaa Hamed Ali*[1]

[1]Department of Microbial Chemistry, Genetic Engineering and Biotechnology Division, National Research Centre, 33 El Buhouth St., EG-12 622 Giza, Egypt

[2]Botany and Microbiology Department, Faculty of Science, Al-Azhar University, EG-11 884 Nasr City, Egypt

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Summary

Thirty isolated fungal strains were screened for lipase production using Phenol Red plates, containing tributyrin as lipidic substrate, and a novel fungus identified genetically as *Curvularia* sp. DHE 5 was found as the most prominent strain. Various agro-industrial substrates were evaluated as inert supports for lipase production in solid-state fermentation. The highest yield of lipase ((83.4±2.2) U/g on dry mass basis) was reported with wheat bran medium after seven days of fermentation at pH=7.0, temperature of 30 °C, 70 % moisture content, inoculum size of $1.27 \cdot 10^7$ spore/mL and 2 % olive oil as an inducer. Supplementation of the medium with 0.05 % KCl as an ion source further increased lipase production to (88.9±1.2) U/g on dry mass basis. The enzyme was partially purified through ammonium sulphate fractionation (40 %) followed by dialysis, and its optimum pH and temperature were reported at 8.0 and 50 °C, respectively, with remarkable pH and thermal stability.

Key words: lipase, *Curvularia* sp. DHE 5, optimisation of culture conditions, agro-industrial residues, lipase characterisation

Introduction

Lipases (EC 3.1.1.3) catalyse the hydrolysis of acyl glycerol to glycerol, mono- and diacylglycerol. In addition, they catalyse under certain conditions the synthesis of esters through transesterification, thioesterification and aminolysis (*1*). Consequently, lipases find application in different industrial sectors, *i.e.* synthesis of biosurfactants, detergent formulations, oleochemical and agrochemical industries, paper manufacturing and pharmaceutical processing (*2*). Lipases occur widely in nature and their presence has been reported in several plants, animals and microorganisms (including bacteria, fungi, yeasts and actinomycetes). However, microbial lipases occupy a prominent place among other biocatalysts due to their high stability towards extremes of temperature, pH, organic solvents and enantioselectivity (*3*). Fungal lipases are generally preferred for industrial application because they are usually secreted extracellularly, which enables simple extraction from the fermentation medium. Among major fungal species capable of producing high levels of lipase are *Fusarium solani* (*4*), *Rhizopus oligosporus* (*5*) and *Trichoderma harzianum* (*6*).

Microbial lipases are produced by both submerged fermentation (SmF) and solid-state fermentation (SSF) (*7*).

*Corresponding author: Phone: +2 010 6245 6850; E-mail: delghonamy@yahoo.com

ORCiD IDs: 0000-0003-4233-5872 (El-Ghonemy), 0000-0002-1630-4928 (El-Gamal), 0000-0001-5698-6727 (Tantawy), 0000-0002-3106-4467 (Ali)

However, SSF is preferred because of its higher yields, lower water output and less energy requirement (*8,9*). Filamentous fungi are considered as the most adapted microorganism for SSF because of their ability to grow on solid substrates as well as their great tolerance to high osmotic pressure (*10*). Hence, there is an ongoing interest in isolation and characterisation of novel fungal strains that produce lipolytic enzymes with novel properties well suited for industrial applications. Therefore, the target of this research was to evaluate the potential of a novel locally isolated fungal strain identified morphologically and genetically as *Curvularia* sp. DHE 5 for alkaline lipase production in SSF. Optimisation of the culture conditions was also investigated for maximum enzyme production in SSF. In addition, partial purification and characterisation of the enzyme were carried out for possible industrial applications.

Materials and Methods

Materials

Tributyrin, Triton X-100, Tween 80 and gum arabic were obtained from Acros Chemical Co., Geel, Belgium. *p*-Nitrophenylpalmitate and Tris-(hydroxymethyl)-aminomethane were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potato dextrose agar medium was purchased from Laboratorios Conda S.A., Madrid, Spain. Wheat bran, wheat germ, sunflower oil cake and safflower oil cake were kindly donated from Arma Food Industries, Cairo, Egypt. Cotton seed waste was kindly donated from Mohamed Abbas cotton seed mill factory, As Sinbillawain, El Dakahleya, Egypt. Jojoba oil cake was kindly donated from Lana Cosmetics Co., Cairo, Egypt. Rice straw, rice husk and rice bran were kindly donated from Alfyoum Co., Cairo, Egypt. Olive oil cake was kindly donated from Borg Al Arab for Industry, Alexandria, Egypt. All the substrates were thoroughly washed with tap water, dried at 80 °C in an air-circulation oven to reduce their moisture content (up to 5 %), then ground in an electric mill (model 4630, 25 000 rpm; Vigor Industrial Co., Ltd, Hong Kong, PR China) to uniform size (no. 6 mesh) and finally stored in plastic bags at room temperature (25 °C) for later use. Different types of oil (sunflower, mustard, olive, coconut, sesame, castor, corn, black seed, almond and flaxseed) were obtained from a local hypermarket, Cairo, Egypt. Buffers used in the present research (KCl-HCl, pH=2–3, citrate-phosphate, pH=3–6, phosphate, pH=6–8, Tris-HCl, pH=7–9, and carbonate-bicarbonate, pH=9–11) were prepared as described by Gomori (*11*).

Microorganism isolation, morphological characterisation and inoculum preparation

In the current work, thirty fungal strains were isolated locally from different soil samples from Mit Ghamr, El Dakahleya, Egypt, using serial dilution plate technique (*12*). The soil sample suspensions were inoculated on potato dextrose agar (PDA) medium containing 5 mg/L of streptomycin at pH=5.0 and incubated at 30 °C for 72 h. Subsequently, the separate and distinct colonies were picked and subcultured onto PDA slants, incubated at 30 °C for 7 days and maintained at 4 °C.

The morphological and cultural characterisation of the highest lipase-producing fungal isolate including shape, size and colony characteristics (colour, shape, surface, elevation and edge) were investigated for identification purpose (*13*). The inoculum was prepared from one-week-old PDA slant. Conidia were scraped and 5.0 mL of sterile saline water containing 0.1 % Tween 80 (by volume) was added to each slant.

18S rRNA gene sequencing and phylogenetic tree

The fungal strain was cultivated in PDA medium at 30 °C for seven days and directly used for the amplification of fungal 18S rRNA gene by polymerase chain reaction (PCR). Two universal 18S rRNA gene primers ITS1 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4 5′-TCCTCCGCTTATTGATATGC-3′ were used. PCR was performed in a final volume of 25 μL composed of 1 μL of fungal culture as template DNA, 12.5 μL of Taq PCR Master Mix (Promega, Mannheim, Germany), containing Taq DNA polymerase 0.5 U/μL, 500 μM of each dNTP, 20 mM of Tris-HCl (pH=8.3), 100 mM of KCl, 3 mM of each $MgCl_2$ and Bromophenol Blue, 1 μL of each primer (10 μM), and 9.5 μL of double distilled H_2O. PCR procedure was as follows: 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 30 s and extension at 72 °C for 2 min, and final extension at 72 °C for 10 min.

PCR products were separated by electrophoresis (MS-MINIDUO MultiSUB Mini, Cleaver Scientific Ltd., Rugby, Warwickshire, UK) in agarose gel stained with ethidium bromide and visualised under UV light to confirm its purity, quantity and size, using Tris/borate/EDTA (TBE) buffer containing 1 μg/mL of ethidium bromide. DNA fragments were extracted from the gel using Core Bio Gel Extraction Kit (RKT13; Chromous Biotech, Karnataka, India) and finally sequenced. The retrieved gene sequences were aligned and compared to the nucleotide sequences of some fungal strains in GenBank database of the National Centre for Biotechnology Information (NCBI) by using Basic Local Alignment Search Tool (BLAST) (*14,15*). Phylogenetic tree was constructed by utilising MEGA v. 4.0 software (*16*).

Determination of aflatoxins

Detection of aflatoxins of lipase-producing fungal strain was performed using high-performance liquid chromatography (1100 HPLC; Agilent Technology, Santa Clara, CA, USA) technique according to AOAC method 971.24 (*17*). A mobile phase including water/acetonitrile/mehanol in ratio 240:120:40 was used (*18*). Data observed were recorded with Millennium Chromatography Manager software (*19*).

Qualitative and quantitative screening

The isolated fungal strains were screened for lipase production using rapid qualitative tributyrin Phenol Red agar plate (TPRA) method (*20*). Chromogenic substrate plates were prepared by using Phenol Red (0.01 %) along with 1 % tributyrin, 0.1 % $CaCl_2$ and 2 % agar adjusted to pH=7.3. Agar wells of 8 mm in diameter were made with the help of sterilised cork borer and loaded with 100 μL of spore suspension ($1.5 \cdot 10^7$ spore/mL) of each fungal strain

under aseptic conditions. The inoculated plates were incubated at 28 °C for 48 h and diameters of the developed yellow zones were measured in mm. The change in colour of phenol from red to bright yellow was used as an indicator of lipolytic activity. The fungal strains that exhibited the highest yellow zones around the colonies indicating lipase production were selected for further studies.

Based on the qualitative analysis test, the fungal strains that exhibited the highest yellow zones were further screened using SSF for quantitative enzyme assay. A mass of 5 g of wheat bran was added into 250-mL Erlenmeyer flasks, containing synthetic oil-based (SOB) medium (up to 70 % moisture) composed of (in g/L): $NaNO_3$ 0.5, $MgSO_4 \cdot 7H_2O$ 0.5, KCl 0.5, KH_2PO_4 2.0, yeast extract 1.0, Bacto™ peptone 5.0 adjusted to pH=6.0 and supplemented with essential olive oil (1 % by volume). After sterilisation (LAC-J0805 autoclave; Daihan Labtech Co., Ltd., Namyangju-city, Kyonggi-do, Korea) at 121 °C for 20 min, the flasks were cooled, inoculated with 1.0 mL of spore inoculum ($1.27 \cdot 10^7$ spore/mL) and incubated (FOC225E incubator; Velp Scientifica Srl, Milan, Italy) at 30 °C for 7 days. After incubation period, 100 mL of sterilised distilled water were transferred to each flask and the mixture was shaken in refrigerated incubator shaker (Innova® 4230; New Brunswick™ Scientific, Scituate, MA, USA) for 1 h at 180 rpm at room temperature. The developed suspension was squeezed through muslin cloth and centrifuged (refrigerated Sigma 3-18KS centrifuge, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at 5000×*g* and 4 °C for 15 min. The obtained supernatant was used for lipase assay.

Lipase production in SmF

One-mL aliquots of spore suspension ($1.27 \cdot 10^7$ spore/mL) were used to inoculate 250-mL Erlenmeyer flasks containing 50 mL of SOB medium. The flasks were incubated at 30 °C on a rotary shaker (Innova® 4230 shaker incubator; New Brunswick Scientific) at 150 rpm for a week. After incubation, the mycelia were harvested by filtration and the culture filtrates were centrifuged (refrigerated Sigma 3-18KS centrifuge) at 5000×*g* and 4 °C for 15 min. The obtained clear supernatant was used for enzyme assay.

Enzyme assay using pNPP substrate

p-Nitrophenyl palmitate (*p*NPP) was used as a substrate for rapid measurement of lipase activity as described by Pera *et al*. (*21*) but with slight modifications. Substrate solution was prepared as follows: 1.0 mL of solution A (40 mg of *p*NPP in 12 mL of isopropanol) was added dropwise with stirring to 19 mL of solution B (0.1 g of gum arabic and 0.4 mL of Triton X-100 in 90 mL of distilled water) and the obtained emulsion remained stable up to 2 h. The enzyme assay mixture, composed of 0.5 mL of Tris-HCl buffer (pH=8.0, 0.1 M), 1.0 mL of the substrate solution, 0.1 mL of suitably diluted enzyme and 1.4 mL of distilled water, was incubated at 40 °C for 15 min. The reaction was terminated by the addition of isopropanol (0.2 mL) and the released *p*-nitrophenol was measured at 410 nm in Cary-100 UV-Vis spectrophotometer (Agilent Technologies, Frankfurt, Germany). One unit of enzyme activity is defined as the amount of lipase that releases one μmol of *p*-nitrophenol per min under the standard conditions. Enzyme activity was expressed in U/g on dry mass basis.

Optimisation of various physicochemical parameters

Four different media were used in the present study, each containing 5 g of wheat bran supplemented with olive oil (1 %) at moisture content of 70 % and adjusted to pH=6.0. Different production media were designed as follows (in g/L): medium I: KNO_3 2.5, KH_2PO_4 1.0, $MgSO_4 \cdot 7H_2O$ 1.0 and casein hydrolysate 20; medium II: Tween 80 5.0 and $(NH_4)_2SO_4$ 4.0; medium III: $NaNO_3$ 0.5, KCl 0.5, $MgSO_4 \cdot 7H_2O$ 0.5, KH_2PO_4 2.0, yeast extract 1.0, Bacto™ peptone 5.0, and medium IV: maltose 50 and Bacto™ peptone 30.

Various agro-industrial substrates (wheat bran, wheat germ, sunflower oil cake, safflower oil cake, cotton seed waste, jojoba oil cake, rice straw, rice husk, rice bran and olive oil cake) were screened for optimal enzyme production in SSF by the highest lipase-producing fungal strain. The chosen substrate was further used in the subsequent studies. Optimum temperature for lipase production was studied by varying the incubation temperatures from 25 to 45 °C.

The influence of initial pH of the production medium on enzyme formation was examined by changing the pH of the medium from 3.0 to 8.0 (using 1 M HCl or 1 M NaOH). The optimal incubation period of enzyme production was investigated by incubating the inoculated media up to 12 days and the lipase activity in the samples was analysed every 24 h as described above. To study the impact of moisture content on enzyme production, the selected substrate was moistened within the range of 33–90 % (by volume per mass). Optimum inoculum volume per mass ratio for enzyme production was evaluated by using 5–60 % of inoculum prepared as mentioned before. A volume of 1 mL of spore inoculum contained $1.27 \cdot 10^7$ spore/mL.

An experiment was designed to test the effect of various types of oil (sunflower, mustard, olive, coconut, sesame, castor, corn, black seed, almond and flaxseed at 1 %) on enzyme production during SSF. The effect of metal ions on enzyme production was tested by adding individually K^+, Na^+, Mg^{2+}, Ba^{2+}, Ca^{2+} and Co^{2+} in the chloride form at 0.05 % (by mass).

Partial enzyme purification

At the end of incubation period, the crude enzyme was extracted as described previously and precipitated by adding various mass fractions of ammonium sulfate solutions (from 20 up to 100 % saturation level) with constant stirring at 4 °C for 24 h. The precipitated proteins were separated by centrifugation at 10 000×*g* and 4 °C for 15 min, suspended in a minimal volume (5 mL) of 0.1 M Tris-HCl buffer (pH=8.0), dialysed against the same buffer (0.05 M) overnight at 4 °C using a dialysis membrane (Visking size 3-20/32, diameter of 15.9 mm and capacity of 1 mL/cm; Medicell Membranes Ltd, London, UK) and examined for lipase activity (fraction with the highest enzyme activity was selected for further studies).

Enzyme characterisation

The effect of pH on the partially purified enzyme was evaluated by monitoring enzyme activity in the pH range of 3–10 (using 0.1 M sodium acetate buffer, pH=3–5, phosphate buffer, pH=6–7, Tris-HCl buffer, pH=7–9 and carbonate-bicarbonate buffer, pH=9–10). The enzyme stability at various pH values was determined by incubating the enzyme (in the absence of substrate) at desired pH for 2 h and assaying the residual enzyme activity as described previously. The activity of non-incubated enzyme was regarded as the control (100 %).

The optimal temperature for lipase activity was measured by conducting the enzyme-substrate reaction at various temperatures (30–90 °C at 5 °C intervals) at pH=8.0 and the concentration of liberated *p*-nitrophenol was measured. Thermal stability behaviour of the partially purified lipase was determined in terms of residual activity after incubation of enzyme at desired temperature (50, 55, 60 and 70 °C) up to 1 h. The activity of the thermally non-treated enzyme was used as control (100 %).

Protein determination

The concentration of protein was determined as described by Bradford (*22*) and bovine serum albumin (BSA) was used as a standard. Bradford reagent was prepared by dissolving 100 mg of Coomassie Brilliant Blue G-250 (Sigma-Aldrich) in 50 mL of 95 % ethanol, then 100 mL of 85 % phosphoric acid were added to the mixture and the resulting reagent was filled to 1 L with distilled water and stored in dark until use. For protein quantification, 0.1 mL of diluted enzyme solution was transferred into a clean test tube, 5 mL of dye reagent were added and the sample was allowed to stand for 5–10 min at room temperature, then measured at λ=595 nm (Cary-100 UV-Vis spectrophotometer; Agilent Technologies, Göttingen, Germany).

Statistical analysis

Data reported represent the mean value of triplicate trials with standard deviation (S.D.). The software used to calculate the data was Excel 2010 (Microsoft Corp., Redmond, WA, USA).

Results and Discussion

Qualitative analysis of lipase production by isolated fungal strains

In the rapid qualitative tributyrin Phenol Red agar plate (TPRA) method, out of thirty fungal strains screened for extracellular lipase activity, seventeen gave positive test result with various degrees depending on the intensity of the produced yellow colour. However, fungus 25A exhibited the largest yellow zone (Fig. 1a; for colour version see www.ftb.com.hr) followed by fungus 27A. Yellow colour can be interpreted as the consequence of pH changes in the media caused by increased level of fatty acids due to their release from triglycerides by the action of lipase. Many investigators reported the validity of the previously mentioned method for the primary screening of lipase from different filamentous fungal strains (*23–25*). However, for further confirmation spectrophotometric quantitative method was performed.

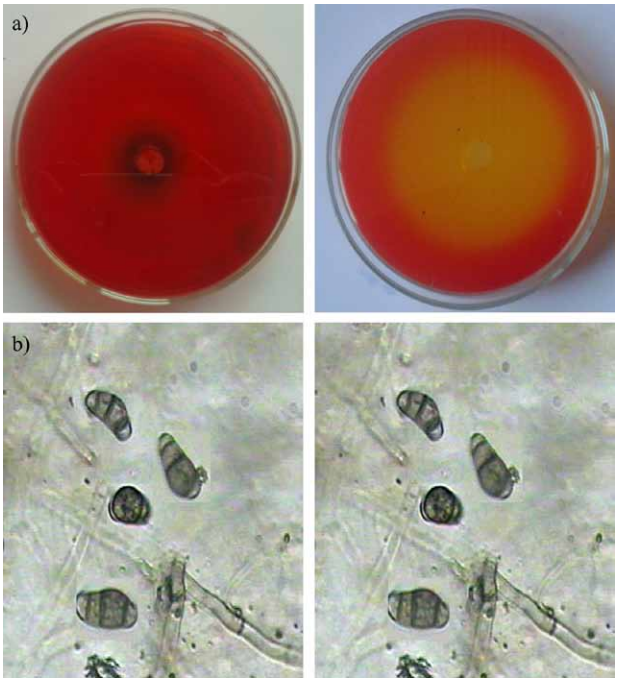

Fig. 1. A novel fungus *Curvularia* sp. DHE 5 (GenBank accession no. KT354967): a) qualitative analysis of lipase production using tributyrin Phenol Red agar plate (TPRA) method; left side=control, right side=agar plate containing tributyrin as substrate with Phenol Red as pH indicator, and b) microscopic examination of *Curvularia* sp. DHE 5; conidiophores were 5 μm in diameter and the conidia were golden brown, or dark reddish brown, broadly ellipsoidal and unilaterally flattened to distinctly geniculate with 4 septa

Quantitative analysis of lipase production by isolated fungal strains

Based on the qualitative screening results, the fungal strains that exhibited the highest yellow zones were further chosen for quantitative analysis using *p*NPP as a substrate. The reported results (Table 1) indicated that almost all selected fungi from the tested set produced extracellular alkaline lipase with different proportions. However, the most potent lipase producer was found to be fungus 25A ((55.9±2.5) U/g), followed by fungus 28A ((44.4±1.9) U/g) and fungus 27A ((39.4±1.2) U/g). The lowest enzyme production was obtained with fungal strains 12A, 2A and 29A, respectively. These results were higher than those reported by Cihangir and Sarikaya (*26*) for extracellular lipase production from *Aspergillus* sp. (17 U/mL) isolated from a soil sample in Turkey. In another study, Kaushik *et al.* (*27*) reported the production of an extracellular lipase from *Aspergillus carneus* with a maximum activity of 13 U/mL. In SSF, Kempka *et al.* (*28*) and Vargas *et al.* (*29*) reported optimum lipase production of 40 and 30 U/g in *Penicillium verrucosum* and *P. simplicissimum*, respectively. It is worth mentioning that fungal strains 5A, 22A and 23A did not show any lipase activity when grown in solid-state fermentation.

Evaluation of aflatoxins and identification of the selected fungal strain

In the present study, toxicity test was performed to confirm the selection of the highest lipase-producing fun-

Table 1. The results of qualitative and quantitative analyses of extracellular alkaline lipase production (on dry mass basis) by locally isolated filamentous fungi

Microorganism	*d*/mm	Lipase activity/(U/g)
Isolate 1A	43	15.9±1.3
Isolate 2A	26	6.7±0.3
Isolate 3A	–	N.D.
Isolate 4A	–	N.D.
Isolate 5A	9.0	0.00
Isolate 6A	–	N.D.
Isolate 7A	–	N.D.
Isolate 8A	16	18.5±1.5
Isolate 9A	–	N.D.
Isolate 10A	–	N.D.
Isolate 11A	–	N.D.
Isolate 12A	18	0.7±0.2
Isolate 13A	30	23.2±1.0
Isolate 14A	–	N.D.
Isolate 15A	–	N.D.
Isolate 16A	–	N.D.
Isolate 17A	–	N.D.
Isolate 18A	–	N.D.
Isolate 19A	29	25.4±1.1
Isolate 20A	16	18.0±1.0
Isolate 21A	11	10.3±0.8
Isolate 22A	10	0.00
Isolate 23A	11	0.00
Isolate 24A	–	N.D.
Isolate 25A	80	55.9±2.5
Isolate 26A	43	18.6±1.1
Isolate 27A	55	39.4±1.2
Isolate 28A	37	44.4±1.9
Isolate 29A	32	7.3±0.7
Isolate 30A	28	8.3±0.7

Data are expressed as mean value±S.D. of triplicate measurements. –=no formation of yellow colour, *d*=diameter of the yellow zone in mm.
N.D.=not detected

gal strain according to AOAC method 971.24 (*17*). The reported results clearly indicated that fungus 25A was a non-mycotoxin-producing fungal strain. Isolate 25A was identified through morphological and culture characteristics as *Curvularia lunata* (Fig. 1b). PCR amplification of 18S rRNA gene, using the primers ITS1 and ITS4, revealed efficient amplification: a single band of 550 bp. According to 18S rRNA gene sequence similarities, the close relatives were *Curvularia lunata* QH-CL1 (99 %), *Curvularia lunata* 3K0205 (98 %) and *Curvularia lunata* GZJZ201302 (97 %). Therefore, the phylogenetic analysis suggests that strain DHE 5 is a new *Curvularia* species and the nucleotide sequences of 18S rRNA genes were deposited to GenBank under the accession number KT354967 (Fig. 2). To the best of our knowledge, no lipases produced by *Curvularia* sp. have been described; therefore, this finding justified the selection of this fungal strain for further studies.

Comparative evaluation of SmF and SSF systems

Extracellular lipase production by *Curvularia* sp. in SSF of (56.2±0.4) U/g on dry mass basis was found to be 7-fold higher than that in SmF using SOB medium ((7.8±0.1) U/mL) (data not shown). The lower enzyme yield when using SOB medium is thought to result from the complexity of the medium components and increased accumulation of intermediate metabolites. Therefore, this study indicates that SSF is more suitable for enzyme production than SmF due to its superior productivity, reduced energy requirements and low wastewater output. Besides, the substrates utilised by microorganisms for enzyme production in SSF are much cheaper than the SmF substrates. Mateos-Díaz *et al*. (*30*) reported extracellular lipase activities of 1500 U/g and 50 U/mL in *Rhizopus homothallicus* cultivated by SSF and SmF, respectively. Furthermore, de Azeredo *et al.* (*31*) obtained lipase activities of 17 U/g and 12 U/mL when *Penicillium restrictum* was cultivated under SSF and SmF conditions, respectively.

Optimisation of different physicochemical parameters for lipase production

Effect of medium composition on lipase production

The physiological studies were started by studying the effect of medium type on extracellular lipase production by *Curvularia* sp. DHE 5. The reported results indicated that medium IV was the best medium for lipase production ((62.2±0.7) U/g), while medium I ((57.1±0.6) U/g) and medium III ((52.3±0.5) U/g) gave moderate lipase yield (data not shown). The lowest enzyme yield ((44.5±0.3) U/g) was observed with medium II, which is in agreement with the observations of Lin *et al*. (*23*). Sarkar *et al*. (*24*) stated that the production of lipase is improved when using complex medium instead of the simple one, while Rodriguez *et al*. (*25*) reported that lipase formation by *Rhizopus hornothallicus* was highly affected by medium constituents besides pH, temperature and inoculum size.

Substrate screening

In the present study, the highest enzyme production (in U/g on dry mass basis) by *Curvularia* sp. was reported when using wheat bran as a medium (62.9±1.3), followed by sunflower oil cake (57.3±0.9), cotton seed waste (56.8±1.1) and jojoba oil cake (54.3±0.7) (Fig. 3). These results are congruent with those observed during enzyme production by *Aspergillus* sp. (*32*) and *Aspergillus niger* (*33*). Among different agro-industrial residues, wheat bran is considered as the universal substrate due to its high nutritional value and low lignin content, which is very helpful for growth initiation and replication of microorganisms. Besides that, wheat bran provides a large surface area because it remains loose even under increased moisture compared to other substrates (*34*).

Incubation period

The impact of time course on lipase production by *Curvularia* sp. DHE 5 grown under SSF using wheat bran

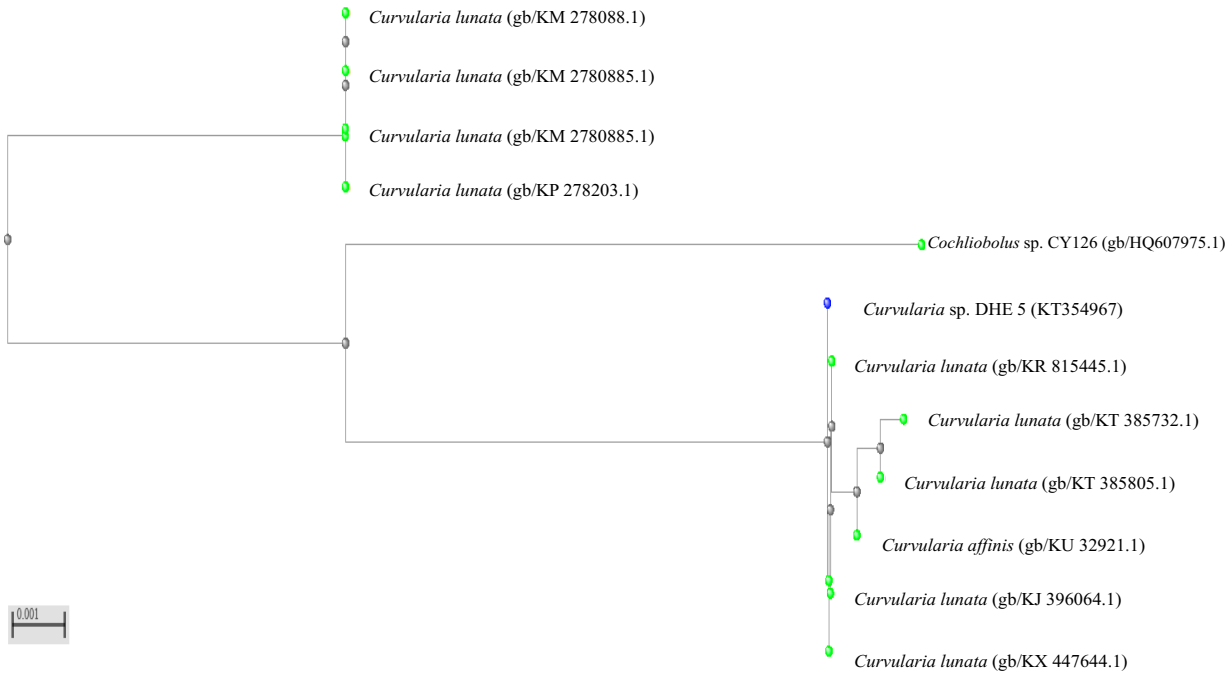

Fig. 2. Phylogenetic analysis of *Curvularia* sp. DHE 5 based on the results of polymerase chain reaction (PCR) amplification of the 18S rRNA gene

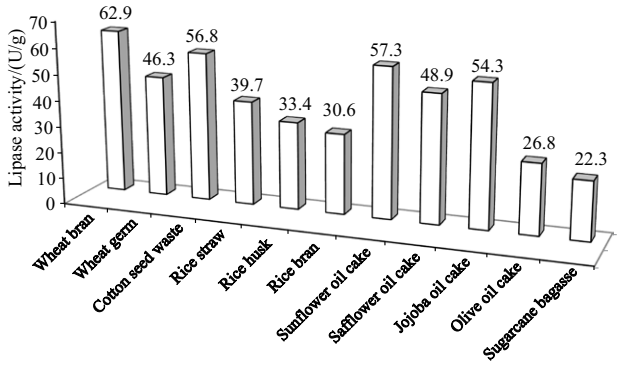

Fig. 3. Influence of various agro-industrial residues on lipase production (on dry mass basis) by *Curvularia* sp. DHE 5

as substrate was evaluated. Results presented in Table 2 show that optimal enzyme production of (63.4±1.0) U/g on dry mass basis was achieved on the seventh day; however, a reduction in enzyme yield was detected with a prolonged incubation period, which might result from the reduction of nutrients or change in pH of the medium or denaturation of the enzyme (*35*). These results are consistent with Rajan and Nair (*36*) who reported that maximum enzyme yield from *Aspergillus fumigatus* was found after seven days of fermentation. Mahadik *et al*. (*33*) observed optimal lipolytic activity of *A. niger* lipase on the fifth day of incubation, while Kamini *et al*. (*37*) and Gutarra *et al*. (*38*) reported that the highest yield of lipase using *A. niger* and *P. simplicissimum*, respectively, was achieved after 72 h of fermentation.

Effect of temperature

Incubation temperature strongly affects the biochemical activities of different microorganisms (*39*). Results shown in Table 2 clearly indicated that the maximal lipase production of (62.4±1.1) U/g by *Curvularia* sp. DHE 5 was observed at incubation temperature of 30 °C after seven days, while a slight decrease in enzyme production was reported at temperatures below optimum. Furthermore, a drastic drop in enzyme yield was noticed at the temperatures above the optimum with least activity detected above 40 °C. These negative impacts might result from the production of proteases at elevated temperatures. Similar results were reported by Rehman *et al*. (*40*) and Zhang and Zeng (*41*) for lipase production by *Penicillium notatum* and *Pseudomonas* sp. 7323, respectively, while the optimal temperatures for lipase formation by *Ganoderma lucidum* (*42*) and *Colletotrichum gloeosporioides* (*43*) were observed at 26.5 and 25 °C, respectively.

Influence of initial medium pH

The initial pH of the growth medium is considered as an imperative parameter and greatly affects the growth and enzyme production by various microorganisms in SSF. However, filamentous fungi are capable of growing in a wide pH range in SSF, because solid substrates have a better buffering capacity (*44*). In this study, maximum lipase production of (71.2±1.1) U/g on dry mass basis by *Curvularia* sp. DHE 5 was reported at initial pH=7 on the seventh day of incubation at 30 °C, while a reduction in lipase yield to (53.4±1.8) U/g (Table 2) was detected when the pH value increased from 7 to 8. Likewise, optimum pH for lipase production by *Penicillium chrysogenum* was observed near neutral value (*45*). Rehman *et al*. (*40*) and Lin *et al*. (*23*) reported that pH=5.5 was the optimal for lipase production in *P. notatum* and *Antrodia cinnamomea*, respectively. On the other hand, our result is lower than that reported for lipase production by *Fusarium solani*, which was pH=8.6 (*46*).

Table 2. Effect of different physical parameters on extracellular alkaline lipase production (on dry mass basis) by *Curvularia* sp. DHE 5 in solid-state fermentation (SSF)

t(incubation)/day	Alkaline lipase activity/(U/g)
3	23.7±0.2
4	40.2±0.5
5	47.6±0.6
6	58.2±0.8
7	**63.4±1.0**
8	54.3±0.8
9	49.6±0.5
10	41.7±0.4
Temperature/°C	
25	48.9±0.5
30	**62.4±1.1**
35	24.5±0.3
40	5.8±0.2
45	1.4±0.2
pH	
3.0	16.6±0.1
4.0	22.5±0.3
5.0	44.2±0.7
6.0	57.4±0.8
7.0	**71.2±1.1**
7.5	67.8±1.1
8.0	53.4±1.8
w(moisture)/%	
33	46.3±0.5
50	54.2±0.6
66	63.4±0.8
70	**72.8±1.0**
75	70.9±1.2
80	59.3±0.8
85	52.5±1.0
Inoculum size/(spore/mL)	
$0.64 \cdot 10^7$	75.6±1.2
$1.27 \cdot 10^7$	**80.8±2.1**
$2.54 \cdot 10^7$	67.4±2.2
$3.81 \cdot 10^7$	55.1±1.0
$5.08 \cdot 10^7$	48.8±0.9
$6.35 \cdot 10^7$	33.4±0.3

Data are expressed as mean value±S.D. of triplicate measurements

Effect of moisture level

In the present investigation, maximum lipase yield of (72.8±1.0) U/g on dry mass basis was reported at 70 % (by volume per mass) moisture content on the seventh day of incubation at 30 °C and initial pH=7.0 (Table 2). However, at higher or lower moisture content, a lower enzyme yield was achieved. At higher moisture content, the lower enzyme yield might result from the decrease of substrate porosity, alternation of substrate particle structure and development of stickiness, which leads to low oxygen transfer and diffusion, while at lower moisture levels the reduction of nutrient solubility results in an improper swelling with a higher water tension that decreased the enzyme yield (*35,44*). Sun and Xu (*44*) and Imandi *et al*. (*47*) reported the same results for lipases produced by *Y. lipolytica* and *Rhizopus chinensis*, respectively. Rehman *et al*. (*40*) investigated optimum lipase production of 3426 U/g by *Penicillium notatum* at 60 % moisture level.

Inoculum concentration

Results shown in Table 2 clearly revealed that maximum lipase production of (80.8±2.2) U/g was obtained with 1.0 mL ($1.27 \cdot 10^7$ spore/mL) of inoculum after seven days of fermentation at 30 °C and pH=7.0. In addition, at higher or lower inoculum size, a negative effect on lipase production was detected. At lower inoculum level, the microbial biomass cannot proliferate quickly; hence, substrate degradation is slow, which subsequently affects the production of metabolites. The inhibitory effect on enzyme production at higher inoculum size might result from the depletion of the nutrients and oxygen available in the growth medium (*48–51*).

Supplementation of wheat bran with various types of oil

Data shown in Fig. 4 clearly indicated that all the tested oil types were suitable for enzyme production but at various extents; however, olive oil was shown to be the best inducer for the production of lipase ((83.1±2.2) U/g) by *Curvularia* sp. DHE 5 using wheat bran as substrate in SSF after seven days of growth at incubation temperature of 30 °C and initial pH=7.0. These results might be correlated with the presence of high percentage of oleic acid (28 %) in olive oil, while the other oils contain higher levels of linoleic acid (30–50 %) (*52*). Iwai and Tsujisaka (*53*) reported that the optimum lipase production was found to be correlated with the higher content of oleic acid in the oil. Similar results were reported for maximum lipase production from *Aspergillus oryzae* (*54*) and *Penicillium wortmanii* (*55*). Adinarayana *et al*. (*56*) observed the optimal production of lipase in *Aspergillus* sp. of 1664 U/g on dry mass basis at 1 % olive oil. Mladenoska and Dimitrovski (*57*) reported maximum lipolytic activity of 0.28 U/mL in *Geotrichum candidum* M2 with sunflower oil.

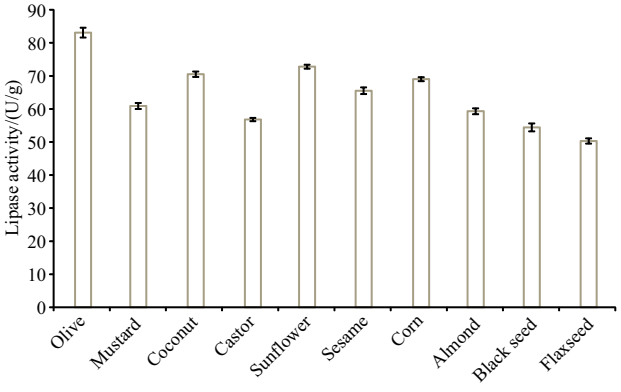

Fig. 4. Effect of different oil sources on lipase production (on dry mass basis) by *Curvularia* sp. DHE 5 in solid-state fermentation (SSF)

The influence of olive oil volume fraction on the production of lipase was evaluated. The highest enzyme yield of (83.4±2.2) U/g was reported at 2 % olive oil (data not shown), while at higher volume fractions a decrease in lipase production was observed, which could be correlated with its effect on aeration rate of the culture that might modify the microbial metabolism and promote a delay in mycelium growth and lipase production as reported by Rao *et al*. (*58*). This result is consistent with that reported by Rajendran *et al*. (*59*) for lipase production by *Candida rugosa*. Teng and Xu (*60*) also reported optimum lipase production by *R. chinensis* at 2.3 % olive oil.

Effect of various metal ions

Metal ions are the important minor nutrients in the medium for cell mass formation and act as cofactor for several biosynthetic enzymes. In the present investigation, several mineral ion sources were incorporated individually in the optimised medium to determine their effect on lipase production by *Curvularia* sp. DHE 5. The obtained results clearly indicated that lipase production was slightly enhanced by KCl ((88.9±1.2) U/g) followed by $MgCl_2{\cdot}6H_2O$ (83.6±0.9) and $CaCl_2{\cdot}2H_2O$ ((82±1.3) U/g), while the lowest enzyme production was observed with $BaCl_2{\cdot}2H_2O$ and $CoCl_2{\cdot}2H_2O$ (Fig. 5). Similarly, Açikel *et al*. (*61*) reported the enhancement of lipase production by *R. delemar* in the presence of metal ions Na^+, K^+, Ca^{2+} and Mg^{2+} by 1.9-, 1.85-, 1.83- and 1.76-fold, respectively. In addition, the production of extracellular lipase from *Aspergillus terreus* was also improved in the presence of Ca^{2+} and Mg^{2+} ions in the growth medium (*62*).

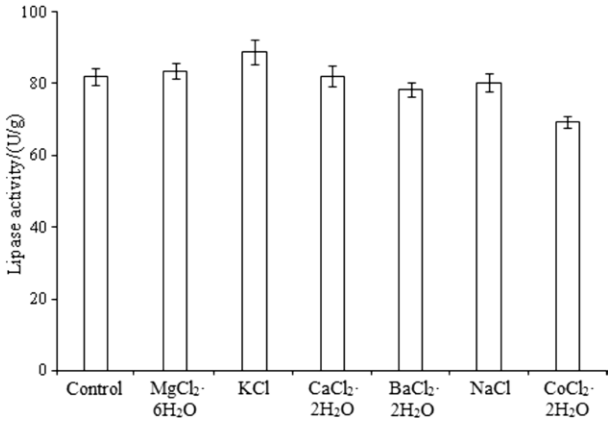

Fig. 5. Effect of various metal ions (γ=50 mg/L) on lipase production (on dry mass basis) by *Curvularia* sp. DHE 5 in solid--state fermentation (SSF)

Partial purification and characterisation of alkaline lipase

In the present study, four fractions were obtained by stepwise addition of ammonium sulphate using four mass per volume ratios, namely 20, 40, 60 and 80 %. Results in Table 3 clearly demonstrated that the purification of alkaline lipase from *Curvularia* sp. DHE 5 through ammonium sulfate precipitation (stepwise precipitation between 20–40 %) and dialysis resulted in 3.1-fold purification with 50.6 % recovery. Besides, the total protein mass decreased from (1858±43) to (309±31) mg, while the specific activity was improved from 8.1 to 24.7 U/mg. Other fractions showed lower specific activities and purification folds, which indicates that more foreign proteins are associated with these fractions.

Optimal pH and pH stability of the partially purified lipase

In the current study, optimum pH for lipase activity was evaluated and the highest enzyme activity was reported at pH=8 using Tris-HCl buffer (0.1 M), as shown in Table 4. Results revealed that the partially purified enzyme could retain more than 90 % of its initial activity in the pH range from 6 to 9, while a reduction in the enzyme activity was detected at pH higher or lower than the optimum. This inhibitory effect might be attributed to the fact that the enzyme is proteinic in nature and hence any vari-

Table 4. Optimum pH of partially purified lipase produced by *Curvularia* sp. DHE 5

Buffer (c=0.1 M)	pH value	Lipase activity/(U/mL)
KCl-HCl	2.0	11.1±0.2
	2.5	12.8±0.2
	3.0	16.9±0.3
Citrate-phosphate	3.0	11.8±0.2
	4.0	14.1±0.2
	5.0	17.8±0.3
	6.0	27.1±0.9
Phosphate	6.0	40.5±1.2
	7.0	98.9±2.5
	8.0	143.1±3.1
Tris-HCl	7.0	114.0±1.8
	8.0	161.0±2.4
	8.5	153.1±2.8
Carbonate-bicarbonate	9.0	114.2±1.8
	9.5	77.2±0.9
	10.0	53.3±0.4

Data are expressed as mean value±S.D. of triplicate measurements

Table 3. Partial purification of extracellular alkaline lipase produced by *Curvularia* sp. DHE 5

Purification step	Total activity	m(total protein)	Specific activity	Purification	Recovery
	U	mg	U/mg	fold	%
Culture filtrate	15083±117	1858±43	8.1	1.0	100
Ammonium sulphate precipitation (20–40 %)	8025±129	354±29	22.7	2.8	53.2
Dialysis	7627±112	309±31	24.7	3.1	50.6

Data are expressed as mean value±S.D. of triplicate measurements

ation in the pH value can profoundly affect ionic character of its amino or carboxylic groups, affecting the conformation of the enzyme. In addition, the affinity between the enzyme and its substrate might be affected by the pH of the reaction. Similarly, Sharma *et al.* (*63*) reported the same result for the activity of lipase purified from *Bacillus* sp.

The pH stability profile showed that the activity of the partially purified enzyme was highly stable at alkaline pH values. The enzyme retained more than 90 % of its initial activity when incubated at pH values from 7 to 9 and about 73 % at pH=11 (Table 5). The lower stability of the enzyme at higher or lower pH values might be attributed to its denaturation, which leads to its inactivation. *Bacillus thermoleovorans* lipase was reported to be highly active at higher pH values and stable in a pH range from 5 to 11 (*64*). Similarly, Saxena *et al*. (*65*) reported that lipase purified from *Aspergillus carneus* was stable up to 24 h at pH=8 to 10.

Table 5. The pH stability profile of the partially purified alkaline lipase produced by *Curvularia* sp. DHE 5

pH	Relative activity/%
3.0	55.9±2.1
4.0	71.4±2.4
5.0	77.1±1.8
6.0	91.5±3.1
7.0	100
7.5	100
8.0	100
9.0	93.6±2.4
9.5	86.7±2.6
10	77.3±1.5
11	72.6±1.4

Data are expressed as mean value±S.D. of triplicate measurements

Optimum temperature and thermal stability

Results shown in Fig. 6 clearly revealed that the optimum temperature for enzyme activity was 50 °C, while a gradual decrease in lipase activity was detected at higher temperatures, which might be attributed to the change in the conformation that causes loss in specificity of the active site (*66*). This result is in accordance with the findings of Liu *et al*. (*67*) for *A. niger* AN0512 lipase. Lima *et al*. (*68*) reported that most of *Penicillium* lipases exhibited optimum activities at temperatures from 25 to 45 °C, except *P. aurantiogriseum* lipase, which has an optimum activity at 60 °C.

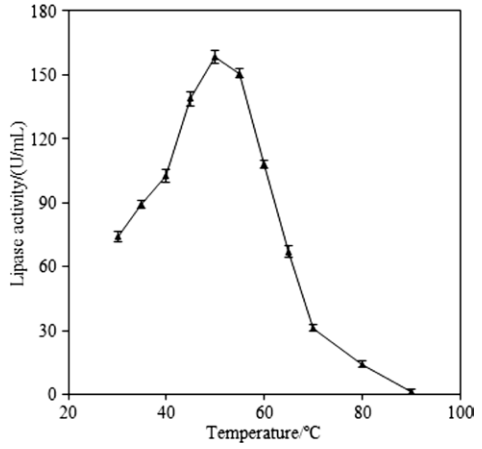

Fig. 6. Temperature optimum of partially purified lipase produced by *Curvularia* sp. DHE 5

Regarding thermal stability behaviour of the partially purified lipase, no significant loss in enzyme activity was detected when the enzyme was preincubated up to 1 h at 50 °C. Moreover, the enzyme retained about 63 % of its original activity after 1 h of incubation at 60 °C (Fig. 7). These results showed that *Curvularia* sp. DHE 5 lipase is more stable than the lipase purified from *Fusarium oxysporum*, which decreased by 50 % at 60 °C after 60 min (*69*). Sethi *et al*. (*70*) reported that lipase purified from *A. terreus* retained about 80 % of its original activity after 60 min at 60 °C.

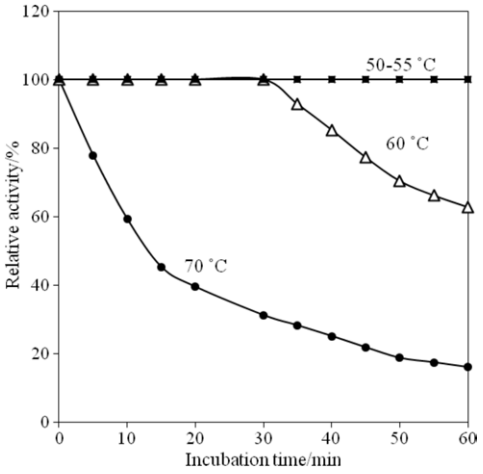

Fig. 7. Thermal stability behaviour of partially purified lipase produced by *Curvularia* sp. DHE 5

Conclusions

Thirty fungal isolates were screened for their ability to produce alkaline lipase in solid-state fermentation (SSF). Fungal isolate 25A tentatively identified as *Curvularia* sp. DHE 5, using 18S rRNA analysis, was proven to be the highest lipase producer when using wheat bran as substrate. Furthermore, optimisation of physicochemical parameters led to 7-fold increase in lipase production with maximum lipase production on dry mass basis of (89±1.2) U/g at incubation temperature of 30 °C, pH=7.0 and 70 % moisture content after seven days of incubation using olive oil (2 %) as an inducer. Optimum temperature and pH of the partially purified enzyme were reported to be 50 °C and pH=8.0, respectively, with remarkable thermal and pH stability.

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