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ORIGINAL ARTICLE

Lipase production by *Aspergillus terreus* using mustard seed oil cake as a carbon source

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Abstract An extracellular lipase-producing fungus was isolated from the garden soil of the Post Graduate Department of Botany, Utkal University, Bhubaneswar, Odisha, India and identified as *Aspergillus terreus*. The *A. terreus* strain isolated was found to be capable of producing lipase in both solid state culture and liquid static surface culture. Experiments aimed at evaluating and improving the production of lipase and at studying the culture conditions revealed that of the many different materials tested as substrates, mustard oil cake (MoC) was the best substrate for extracellular lipase production. A correlation was found between the lipase production profile and biomass development. In a study aimed at continuing this line of research, we have investigated the influence of various culture conditions, such as environmental (i.e. temperature and pH), nutritional (i.e. carbon, nitrogen, metal ions, vitamins, combined agro-wastes and growth regulators) and other factors (inoculum size and initial moisture content) on the production of lipase by *A. terreus* in solid state and liquid static surface cultures. We observed that optimum lipase biosynthesis occurred under the following conditions: initial pH of 6.0, 30 °C, a 96-h incubation, lactose and ammonium persulphate as the carbon and nitrogen source respectively and 80 % moisture content. Changes in the vitamins (vitamin C, riboflavin, folic acid and vitamin E) and growth regulators (gibberellic acid, kinetin, 6-benzylaminopurine and 2,4-dichlorophenoxyacetic acid) did not support enhanced lipase production. MoC and neem oil cake (NoC) added to the media at a ratio of 9:1 respectively, supported maximum lipase production. Based on these results, we concluded that controlling the various culture conditions, supplementing MoC as a substrate and nutrient source modification of the medium can spectacularly enhance lipase biosynthesis by *A. terreus*.

Keywords *Aspergillus terreus* · Lipase · Mustard oil cake · Nutrient source modification · Solid state fermentation

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Introduction

Lipases (triacylglycerol acylhydrolases, EC: 3.1.1.3) are ubiquitous enzymes, biosynthesized by a variety of animals, plants and microorganisms, but the majority of lipases implemented for biotechnological purposes are produced by bacteria and fungi (Sztajer and Maliszewska 1988). The microbial extracellular lipases are commercially significant because of their low production cost, greater stability and wider availability relative to those of plant and animal origin. The most industrially important lipases of fungal, mold or bacterial origin are extracellular and can be produced in economically feasible systems. Fungi are the preferable source of enzymes as they secrete copious amount of enzymes extracellularly, thereby facilitating effortless and simple extractions from different fermentation media (Jaeger et al. 1999). Lipases are members of a vital enzyme community owing to their notable levels of activity and

stability in both aqueous and non-aqueous media, a property which enables them to perform several catalytic reactions, such as acidolysis, alcoholysis, aminolysis, esterification and transesterification (Gutarra et al. 2005). Lipases are members of the serine hydrolase enzyme superfamily and are mainly dependent on factors relating to the fatty acid such as its position in the glycerol backbone, its chain length and its degree of unsaturation. Due to the catalytic behavior and specificity of these enzymes, they are recognized as being some of the most important biotechnologically relevant enzymes that have been identified in the last two decades. They are successfully used in various industrial products and processes, such as in food, flavor enhancers, pharmaceuticals, detergents, oleo-chemicals, cosmetics, agriculture and the leather and tea industries (Cao et al. 1997). However, the sole biggest market for their exploitation is in detergents where they very actively function for the removal of fatty residues in cloth washers, dishwashers and clogged drains (Bjorkling et al. 1991).

The most tiresome task in optimizations of culture conditions is the presence of interactive effects of medium components and various cultural factors. The conventional approach taken to optimize media, that of testing one factor at a time is labor-intensive and time-consuming. Lipases are considered to be members of the “high-flying” group of industrial enzymes as they can perform over a wide range of pH and temperature, possess high specificity, do not require cofactors and can catalyze a wide range of reactions. Lipase can be produced in both liquid static surface culture and solid state fermentation (SSF) systems (Huang et al. 1995), but solid state fermentation systems offer many advantages over submerged fermentation (Holker et al. 2004). The SSF process is of special economic interest for countries which have an abundance of biomass and agro-industrial residues dumped as wastes. Further, lipase biosynthesis by novel fungi is found to be affected by many factors, such as pH (Ferreira Costa and Peralta 1999), temperature (Ferreira Costa and Peralta 1999), carbon sources (George et al. 1999) and nitrogen sources (George et al. 1999). Lipase activity most often depends on the availability of large surface area and demands extreme mild conditions. Several structural studies on lipases have provided clues to their mechanism of hydrolytic activity, interfacial activation and stereoselectivity (Pandey et al. 1999). Lipase biosynthesis is generally organism specific and the lipase is released during the late logarithmic or stationary phase (Sharma et al. 2001). Lipase biosynthesis has been achieved by a variety of microorganisms, but the main representative is *Yarrowia lipolytica* (Zvyagintseva 1971; Kamzolova et al. 2011). Other potent lipase producers are widely distributed throughout the fungal kingdom and are the focus of much biotechnological interest in both research and industrial applications. These include *Aspergillus niger, A. carneus*, *Candida cylindracea*, *Humicola lanuginosa*, *Mucor miehei*, *Rhizopus arrhizus*, *R. delemar*, *R. japonicus*, *R. niveus* and *R. oryzae* (Aravindan et al. 2007).

In the study reported here, we attempted to determine the optimum conditions required for the maximum lipase production by *A. terreus* in solid state flask cultures. The optimization of the SSF system was based on the medium composition and establishment of cultural conditions. However, augmenting the productivity of lipase in solid state culture may be of great benefit because the lower production cost of lipase could lead to new industrial applications.

Materials and methods

Substrates and chemicals

Samples of mustard oil cake (MoC), neem oil cake (NoC), groundnut oil cake (GnoC), black gram peels (BGP), green gram peels (GGP), wheat bran (WB), finger millet (FM), banana peels (BP), and orange peels (OP) were obtained from the local market of Bhubaneswar, Odisha, India. Each of these materials was brought to the laboratory in new steel container(s). The samples were dried under observation at 60 °C for 48 h or more until the moisture content was reduced. Cooled samples were later subjected to grinding in a blender and kept under sterile containers until required. Olive oil was purchased from local retail outlets in Bhubaneswar. All of the chemicals used in this study were of AR grade and purchased from Hi-Media Limited, SRL Pvt. Limited and Merck India Limited (Mumbai, India).

Microorganism and inoculum

Aspergillus species, a natural isolate obtained from garden soil of the Post-Graduate Department of Botany, Utkal University, Bhubaneswar, Odisha, India was used for all experiments described herein. The culture was grown on potato dextrose agar (PDA) slants at 30±1 °C for 7 days and stored at 4±1 °C until further use. The fungal isolate was characterized and identified by the National Center for Fungal Taxonomy, New Delhi, India and identified as *Aspergillus terreus* (NCFT 4269.10).

Seven-days-old PDA slant cultures were suspended in 5.0 ml of sterile deionized water and 1.0 ml of the spore suspension was used as the inoculum for pre-culture. Further incubation at 30±1 °C for 1 week was conducted to yield a microbial density of about 5.0×10^8 cells/ml. The cell suspension was used as the inoculum for both screenings (liquid static surface culture and solid state fermentation) at an initial cell density of 1×10^7 cells/ml.

Screening of agro-substrates for lipolytic activity

For screening in liquid static surface culture fermentation (LSSC) condition, 10-g residues were ground to powder, cooked with 100 ml distilled water for at least 15 min and then allowed to cool. After cooling, the boiled residues were filtered and the extract used in the preparation of medium for the LSSC without adjustment of the pH. Sterilized fermentation medium (50 ml) containing either MoC, NoC, GnoC, BGP, GGP, WB, FM, BP or OP as substrates, was put into 150-ml Erlenmeyer flasks and inoculated with 1×10^{7} cells/ml from 7-days-old *A. terreus* culture in broth and incubated at 30±1 °C under static conditions. After 96 h, the samples were processed for the lipase activity assay. In brief, 1 ml of culture was centrifuged at 10,000 rpm at 4 °C for 10 min to obtain a cell-free supernatant. Prior to performing the lipase activity assay (Dheeman et al. 2010), the clear supernatant was filtered through Whatman No. 1 filter paper.

For the SSF system, the agro-industrial residues were sieved to obtain fine particles, washed three times with double distilled water and then oven dried at 80 °C for 48 h. Erlenmeyer flasks of 250-ml capacity containing 5 g (w/w) of the MoC as substrate and 8 ml of minimal salt medium (MSM) solution were shaken to thoroughly mix the contents, the moisture content was adjusted up to 80 % and the flasks were then autoclaved for 15 min at 121 °C (15 psi pressure). This fermentation medium was then inoculated aseptically with the optimum number of spore inoculum and incubated for 4 days at 30±1 °C with intermittent observation.

Enzyme extraction

After the completion of fermentation, the enzyme was extracted from the fermented media (SSF) according to the method of Alva et al. (2007). Briefly, 50 ml of cold 0.1 M phosphate buffer (pH 6.5) was added to the SSF cultures and the mixtures shaken manually for 30 min at 30 °C. The mixture was then filtered through cheese cloth and centrifuged at 8000 rpm at 4 °C for 15 min to remove the spores and other insoluble material. The supernatant was filtered three times through Whatman No. 1 filter paper and the filtrate used as the crude enzyme. Similarly, the centrifuged fermented broth from the LSSC was filtered three times through Whatman No. 1 filter paper and the filtrate used as the crude enzyme. The enzyme obtained from both SSF and LSSC systems was kept in the deep freezer at −20 °C until use in further experiments.

Determination of cell dry weight

Fermented media (LSSC fermentation) were filtered first through cheese cloth then through Whatman No. 1 filter paper. The dry weight of the biomass was determined after drying at 80 °C in a hot air oven (Wadegati Instruments Ltd., Mumbai, India) for up to 24 h. The filtrate was also preserved at 4 °C for further analysis.

Analytical methods

Total protein determination

The total protein content of the crude enzyme extract was determined by the method of Lowry et al. (1951) taking bovine serum albumin as the standard and was expressed as micrograms of total proteins per milliliter of the crude enzyme extract obtained after fermentation.

Lipase assay

Lipase activity was determined by the olive oil substrate emulsion method as per Mustranta (1992). The enzyme extract (0.2 ml) was added to the assay mixture, which consisted of 1.0 ml of substrate emulsion (70.0 ml emulsifying reagent with 30.0 ml olive oil, homogenized for 5 min), 2.0 ml emulsification reagent (17.9 g NaCl, 0.41 g KH_2PO_4, 540.0 ml glycerol, 10.0 g gum arabic and distilled water to a total volume of 1.0 L), and 0.8 ml of 0.2 M potassium phosphate buffer (pH 7.0). This mixture was incubated at 37 °C for 30 min. The reaction time was finalized after the initial rate determination. These conditions were referred to as the standard conditions. The reaction was interrupted by the addition of 2.0 ml of acetone–ethanol mixture (1:1 v/v) so as to promote the extraction of the free fatty acids. The amount of fatty acid liberated was determined by titration with 0.01 N NaOH up to a final pH of 11.0. The reaction blank was prepared by mixing the acetone–ethanol mixture prior to the addition of enzyme extract. One lipase enzyme unit was defined as the amount of enzyme required for the release of 1 μmol fatty acid per minute under the assay conditions. Enzyme activity was expressed as units (U) per milliliter of enzyme extract.

Effect of various process parameters on lipase production

To optimize a standard protocol for enhanced lipase production by *A. terreus*, we evaluated various parameters of SSF, including different pH (3–10), temperatures (24–45 °C), incubation time (24–168 h), and initial moisture content (20–100 %), with different carbon sources (1 %, w/w), nitrogen sources (organic and inorganic) (1 %, w/w), amino acids (1 mM), metal ions (1 mM), antioxidant vitamins (10–50 mg/100 ml), inoculum sizes (2–10 %), and growth hormones (0.0025 mg/g, w/w) and with combined agro-wastes. These parameters were studied to evaluate the optimum conditions required to develop an efficient and cheaper protocol for both the production and recovery of lipase.

Results and discussion

Screening of agro-wastes

Most of the lipases of microbial origin are extracellular and excreted through the cell membrane into the culture medium. Various operating strategies have been evaluated to improve the yield of these enzymes. Many microorganisms, such as *Penicillium restrictum* (Friere et al. 1997), *Aspergillus terreus* (Gulati et al. 1999), *Mucor hiemalis f hiemalis* (Hiol et al. 1999), *Penicillium citrinum* (Miranda et al. 1999), *Rhizopus oryzae* (Hiol et al. 2000), *Cryptococcus* sp. S-2 (Kamini et al. 2000), *Rhizopus arrhizus* (Elibol and Ozer 2000), *Penicillium cyclopium* (Vanot et al. 2001), *Geotrichum* sp. (Kamimura et al. 2001), *Fusarium solani* (Maia et al. 2001) and *Yarrowia lipolytica* (Kamzolova et al. 2011), are also used for lipase production. According to Yadav et al. (1998), *A. terreus* produces an extracellular thermostable lipase when olive oil is used as a carbon source. Therefore, in our study, olive oil was used as a control for lipase production in the LSSC system. However, as olive oil is an expensive component of any economic lipase production medium, we investigated potential candidate replacements of agro-residual waste origin (MoC, NoC, GnoC, BGP, GGP, WB, FM, BP, and OP). Of the various agro-wastes tested, MoC was found to be the best substrate for optimal lipase production in both fermentation processes. Lipase activity was also supported by GnoC and FM, but reduced activity was observed with NoC, GGP and WB as compared to the control (Table 1). In most cases, the SSF system was dominant over LSSC fermentation. Among all of the substrates tried, the highest ratio of polyunsaturated to saturated fatty acids was present in MoC relative to the control (olive oil), which is the cause of maximum biosynthesis of lipase (Johnson and Saikia 2009). Table 1 shows that there is no exact correlation between biomass and lipase excretion. Similarly, Mala et al. (2007) established a SSF for lipase production by *Aspergillus niger* MTCC 2594 using WB and gingelly oil cake as substrates. Papanikolaou et al. (2007) and Darvishi et al. (2009) developed an efficient protocol for lipase production in *Yarrowia lipolytica* by using industrial derivatives of tallow and plant oils respectively as substrates and found that lipase biosynthesis was notably affected by the medium composition and the type of fat/oil. Similar results have been obtained using model microorganisms, such as *Rhodotorula mucilaginosa*, *Lodderomyces elongisporus*, *Penicillium*, *Aspergillus*, and *Fusarium* strains by culturing them on different lipid-containing substrates, such as sunflower and olive oil (Wang et al. 2007; Fakas et al. 2010; Papanikolaou et al. 2011). Alkan et al. (2007) also studied the effect of several agro-industrial residues (wheat bran, rice husk, lentil husk, banana waste, watermelon waste and melon waste) on lipase production by SSF using *Bacillus coagulans*. Mahanta et al. (2008) reported lipase production by SSF with *Pseudomonas aeruginosa* PseA using Jatropha seed cake. Lipase obtained using SSF has been more intensively studied because of its economic importance. Similar criteria are shared by one other research group who have related to their work on lipase production by *Penicillium restrictum* using submerged fermentation (SMF) and SSF (Castilho et al. 2000). Several enzymes are produced by *Aspergillus* species (Ghildyal et al. 1991) and also by other bacterial and fungal species by SSF, but only a few reports on lipase production by the solid-state and SMF systems are available using *Aspergillus terreus*. Lipase activities associated with the cells of *A. terreus* were found to be induced and enhanced by the presence of a lipid substrate in the fermentation medium (Large et al. 1999). Lipid-rich agro-industrial wastes provide an alternative source of carbon as substrates and may help in solving some pollution tribulations, which otherwise might be caused by their dumping. The economics of enzyme production using inexpensive raw materials may result in enzymatic processes being economically competitive with chemical ones (Miranda et al. 1999).

Effect of initial pH on lipase production and mycelial growth

The initial pH of the culture broth was found to be one of the most critical environmental parameters affecting both growth and enzyme production by *A. terreus*. The optimization of process parameter study revealed that maximum lipase production occurred at an initial medium pH of 6.0 followed by 5.0 and 7.0, although the organism was able to grow in a pH range of 3.0–10.0 (Fig. 1); maximum biomass was attained at pH 6.0. Lipase activity and biomass decreased significantly at the alkaline and acidic pH of 9.0 and 5.0, respectively. However, the final pH of the fermentation medium was always lower than the initial pH (data not given). These results are in agreement with those of other studies on lipase production in SSF (Christen et al. 1995; Gombert et al. 1999) and LSSC (Pimentel et al. 1994; Freire et al. 1997) systems using different culture media. The optimum pH in our study (6.0) is significantly lower than those of other lipase producing organisms, such as *Candida rugosa* (pH 7.0) and marine bacterial lipase (pH 10.0) (Camargo de Morais et al. 1998). Furthermore, the metabolite profile of the fungus seems to be non-polar, such that any electrophilic or nucleophilic extrusion from the fungus would definitely interfere with pH. We verified that lipase from *A. terreus* showed a better stability in the pH range of 4.0–8.0.

Effect of temperature

Liquid static surface culture was carried out at temperatures ranging from 24 to 45 °C (steps of 3 °C) at a constant pH of

Table 1 Lipase activity of *Aspergillus terreus* in liquid static surface culture and solid state culture systems using various agricultural wastes as the substrate

Fermentation medium	Cultivation regimen[a]	Enzyme activity (U/ml)	Biomass (g/50 ml)
Mustard oil cake (MoC)	Liquid static surface culture	1366.67±116.66 b	0.203±0.013 b
	Solid state	1566.67±133.33 a	-
Neem oil cake (NoC)	Liquid static surface culture	83.33±16.66 g	0.094±0.007 d
	Solid state	183.33±44.09 e	-
Groundnut oil cake (GnoC)	Liquid static surface culture	1116.67±160.09 c	0.243±0.002 a
	Solid state	833.33±60.09 d	-
Black gram peels (BGP)	Liquid static surface culture	100.00±100.00 f	0.085±0.002 d
	Solid state	133.33±72.64 f	-
Green gram peels (GGP)	Liquid static surface culture	16.66±6.52 i	0.157±0.004 c
	Solid state	50.00±50.00 h	-
Wheat bran (WB)	Liquid static surface culture	200.00±200.01 e	0.080±0.001 d
	Solid state	50.00±50.53 h	-
Finger millet (FM)	Liquid static surface culture	966.66±133.03 d	0.032±0.004 de
	Solid state	766.66±133.03 d	-
Banana peels (BP)	Liquid static surface culture	0	0.017±0.044 e
	Solid state	0	-
Orange peels (OP)	Liquid static surface culture	0	0.031±0.065 de
	Solid state	0	-
Control[b]	Liquid static surface culture	300.00±28.86 e	0.036±0.006 de

Data are presented as the mean ± standard deviation (SD) of replicates (n=3). Values in the same column followed by different lower-case letters are significantly different between treatments and the control at $P \leq 0.05$

[a]The liquid static surface culture (LSSC) and solid state culture experiments were performed for 96 h at 30 °C

[b]Control refers to the basal medium with 1 % (v/v) olive oil to induce lipase biosynthesis

6.0 with 1×10^7 spores/ml as inoculum for 96 h to evaluate the effect of incubation temperature on fungal growth and enzyme production. Maximum enzyme and biomass production were observed in the mesophilic range at 27–36 °C (Fig. 2). Maximum enzyme activity was observed at 30 °C with all other conditions kept constant. Lipase and biomass production gradually increased with increases in temperature up to 30 °C, significantly decreasing thereafter. The results in our study demonstrate that temperature control during the LSSC process was a critical factor significantly affecting growth and lipase production, which correlates with the finding of Lin and Ko (2005). The optimal temperature determined in our study for maximum lipase synthesis and biomass production by *A. terreus* (30 °C) is comparable to those reported for *A. flavus* USM A 10 (Pau and Omar 2004) and *Fusarium glogulosum*. Lima et al. (2003) also studied the effect of temperature on lipase production using *Penicillium aurantiogriseum* and reported that the optimum temperature for lipase production was 29 °C. Cultures for the production of lipases by the genus *Penicillium* are generally incubated at between 25 and 30 °C, most often at 28 °C (Freire et al. 1997).

Effect of incubation time

Maintaining the optimal pH (6.0) and temperature (30 °C), we then standardized the incubation period for lipase synthesis by *A. terreus* in fermentation medium. The effect of incubation period was monitored by measuring lipase

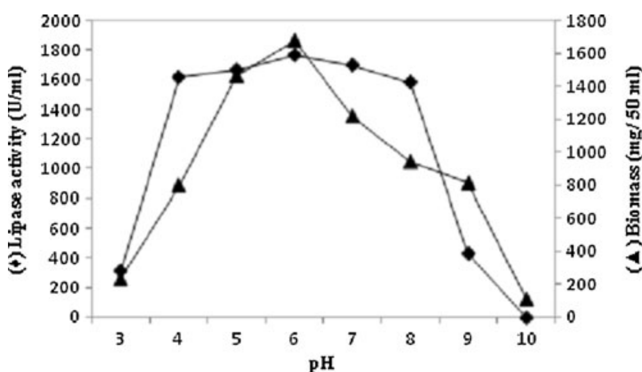

Fig. 1 Effect of pH on lipase production (*filled rhombus*) and mycelial growth (*filled triangle*) in liquid static surface culture (LSSC) by *Aspergillus terreus* after 4 days of cultivation with the initial pH varying from 3.0 to 10.0

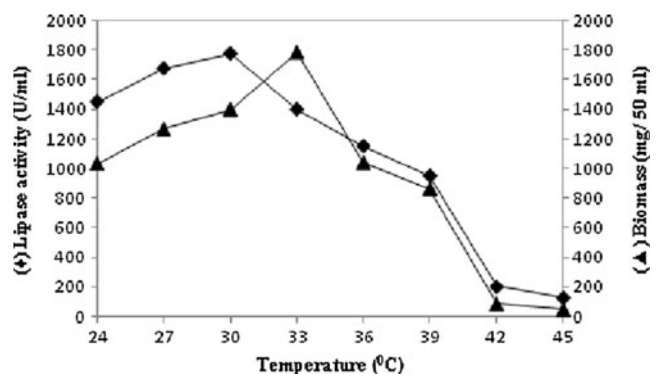

Fig. 2 Effect of temperature on lipase production (*filled rhombus*) and mycelial growth (*filled triangle*) in LSSC by *A. terreus* after 4 days of cultivation at temperatures ranging from 24 to 45 °C in steps of 3 °C

activity and dry biomass. Significant lipase production commenced at 24 h and reached a maximum at 96 h. Further incubation after 96 h did not lead to an increase in lipase activity, only to a slow decrease in lipase activity (Fig. 3). Biomass had substantially increased after 24 h and continued to do so up to 144 h past the maximum enzyme production optimum (Fig. 3) at 96 h, staying stable thereafter before showing a decrease in growth. Lipase production was related to medium pH which dropped relative to the initial pH at all time points. The pH of the culture broth became slightly acidic, reaching to 5.9 at 96 h when lipase activity was at its maximum. The increase in lipase activity in the fermentation medium was proportional to that of the biomass produced up to 96 h, indicating that the lipase production is associated to the growth process, at which time the culture pH was around 5.0–6.0. The decrease in lipase production after long fermentation times could be due to inactivation of the enzyme by extracellular proteases, as has been observed for other lipase-producing microorganisms (Sanchez et al. 1999). Similarly, Kamzolova et al. (2005) reported that lipase biosynthesis was at its maximum during the exponential growth phase, gradually decreasing at the late logarithm phase of growth due to the production of citric acid and nitrogen shortages in the medium. A similar trend has also been reported by Papanikolaou et al. (2007) in *Yarrowia lipolytica*, who concluded that lipase production was significantly reduced in the stationary phase of growth. Swift et al. (1996) reported that once cell densities have reached to a certain threshold level, generally in the late logarithmic phase, the expression of genes encoding exoproteins and the secretion system are induced.

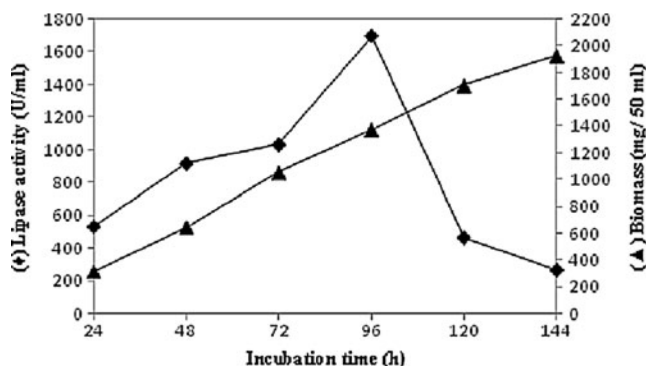

Fig. 3 The incubation time profiles of lipase production (*filled rhombus*) and mycelial growth (*filled triangle*) during LSSC by *A. terreus* using fermentation medium containing mustard oil cake (MoC)

Effect of additional carbon source

The production of lipase and biomass was not, in most cases, significantly enhanced with the addition of sugars to the fermentation medium (Fig. 4). The fungus was able to produce lipase when using each of the carbohydrates as carbon sources, but the addition of lactose resulted in the highest activity, as did the addition of glucose. Sucrose, starch, mannitol, cellulose and maltose were found to be comparatively adequate in terms of enhancing lipase production relative to the control, whereas the addition of fructose and mannose did not significantly enhance lipase production as compared to the control. In most cases, there was little or no difference between these substrates in terms of lipase-promoting property, which suggests that most carbohydrate types are not suitable for lipase production, possibly due to their low availability relative to those present in the medium or because they were preferentially used before lipase production (Rodriguez et al. 2006). Most of the sugars used resulted in decreased lipase production as well as in low growth, which is in accordance with the findings of Gulati et al. (1999). However, it should be noted that similar results have been reported for the *C. rugosa* strain when it was fermented in different concentrations of olive oil and glucose (Valero et al. 1991). Although lipid-based carbon sources seem to be generally essential for obtaining a high enzyme yield, some authors have reported good results in the absence of fats and oils (Lin and Ko 2005; Lin et al. 2006). Kamzolova et al. (2011) reported that a higher concentration of lipid-based carbon sources, such as glycerol did not enhance the lipase biosynthesis by *Yarrowia lipolytica*. The inhibitory action of glycerol on lipase activity has also been reported for *Candida rugosa* (Del Rio et al. 1990).

A range of different carbon sources (i.e., carbohydrates, alcohols, acids, lipids) has been reported to support both the growth of lipolytic enzyme producers and lipase/esterase production (Benjamin and Pandey 1996; Gulati et al. 1999). Therefore, the suitability of different carbon source additives for lipase production by *A. terreus* was investigated (Fig. 4).

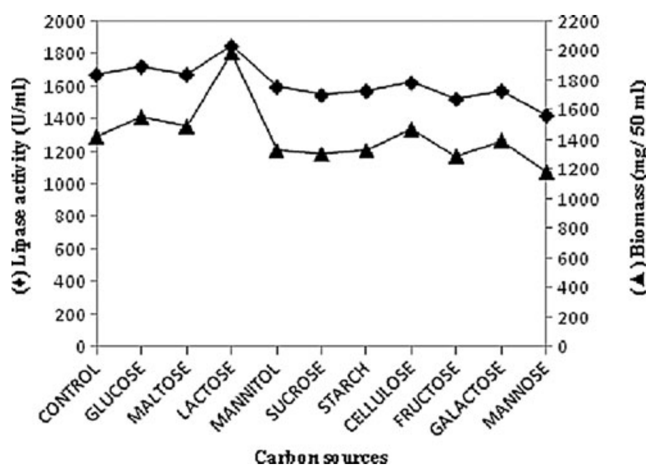

Fig. 4 Effect of carbon source on mycelial growth (*filled triangle*) and lipase production (*filled rhombus*) by *A. terreus* during a 4-days incubation at 30 °C. *Control* Basal medium in the absence of any added carbon source

The addition of most of the sugar additives to the fermentation medium did not have a positive effect on the growth and lipase production by *A. terreus*. A decreased synthesis of lipolytic enzymes was obtained with starch, which is similar to the effect of starch on lipase production in *Issatchenkia orientalis* (Costas et al. 2004). An increase in lipase activity was reported for a mutant strain (UV-10) of *Aspergillus niger* NCIM 1207 when glucose was added to the production medium in the presence of olive oil (Mahadik et al. 2004), which is not in accordance with our findings. Pau and Omar (2004) have also reported that optimum lipase and biomass were attained when the solid substrate was supplemented with 1 % (w/v) lactose using *A. flavus* USM A 10.

Effect of added nitrogen source

Different nitrogen sources were added to the LSSC at a 1 % (w/v) concentration and investigated their suitability for use as additives. Proteins, ammonium, sodium salts, and amino acids were used as additives. Table 2 presents the variations in lipase production observed following the addition of the various nitrogen sources. Lipase production and biomass were highest with beef extract and yeast extract as organic nitrogen sources followed by bacto-peptone (Table 2). However, none of the nitrogen sources supported enhanced lipase production relative to the control. Significant biomass was also produced with beef extract and yeast extract as an organic nitrogen source. This result shows a linear proportion between biomass and enzyme production. Complex nitrogen sources, such as yeast extract, peptones, soybean meal, and corn steep liquor have traditionally been used for lipase production (Sharma et al. 2001). These results differ from the general observation that microorganisms achieve high yields of lipase when organic nitrogen sources are used. Freire et al. (1997) compared different nitrogen sources for lipase production by *Penicillium* strains in SSF and found peptone to give the best results, which also differs from our findings. Likewise, higher lipase production has been reported using yeast extract as a nitrogen source for *Saccharomyces cerevisiae* (Shirazi et al. 1998). Of the different inorganic nitrogen sources tested and among the various ammonium ion-based additives, only $(NH_4)_2S_2O_8$ was found to produce a significant increase in lipase and biomass production (Table 2). This result could possibly be attributed to the indispensable ions (NH_4^+ and S_2O_8), which promote lipase and biomass production, whereas the other ions resulted in significantly reduced lipase activities. The addition of NH_4NO_3 and urea resulted in the greatest decrease in lipase activity. In contrast, Lima et al. (2003) found that lipase production in *Penicillium aurantiogriseum* was stimulated by ammonium sulphate. Lipase activity and biomass production were significantly reduced by urea. Urea was also reported to be inhibitory for lipase synthesis by *Penicillium camemberti* Thom PG-3 (Tan et al. 2004). However, Benjamin and Pandey (1996) reported urea to be an optimal nitrogen source for lipase production in liquid cultures of *Candida rugosa*. Pau and Omar (2004) reported that among all of the inorganic nitrogen sources tested in their study, only ammonium salt had a significant effect on lipase production, which is in agreement with our findings. We tried 17 amino acids and found that maximum activity was attained at a 1 mM concentration of isoleucine, followed by proline and glycine, respectively (Table 2). Maximum biomass was obtained with a 1 mM concentration of proline. However, no amino acids supported higher lipase production in comparison to the control. Based on these results, we conclude that *A. terreus* strain does not require any growth factors, as also stated by Rodriguez et al. (2006) for *Rhizopus homothallicus* IRD 13a. However, Lin et al. (2006) reported a higher value of lipase activity when the culture medium was supplemented with L-asparagine by the edible basidiomycete *Antrodia cinnamomea*.

Effect of metal ions as additives

Metal ions may also play an essential function in lipase production. In our study, we found that Ca^{2+} and Mg^{2+} significantly enhanced lipase production, while the other metal ions tested had an inhibitory effect on lipase production (Table 2). Several authors have also reported a stimulatory effect of calcium, magnesium and iron ions on lipase production by different organisms (Kanwar et al. 2002; Lin et al. 2006). Gulati et al. (1999) reported similar findings to those reported here for lipase production by *A. terreus*. In both cases, there was no noticeable effect on mycelial growth because free Ca^{2+} and Mg^{2+} ions control the exocytosis of proteins in eukaryotes through 'regulated secretion' pathways (Hoshino et al. 1991). Tan et al. (2004) also reported that Mg^{2+}, Na^+ and K^+ ions influence lipase production in *Candida* sp. and concluded that they are also beneficial for biosynthesis of lipase. In their study of *Antrodia cinnamomea*, Lin et al. (2006) found that the highest yield of lipase was achieved when the medium was enriched with Ca^{2+}, Mg^{2+}, Fe^{2+}, Na^+, or K^+. In contrast, Janssen et al. (1994) reported that lipase production by a thermophilic *Bacillus* sp. increased several fold when magnesium, iron and calcium were incorporated into the production medium. Similarly, Ca^{2+} in the presence of Mg^{2+} has been reported to produce a significant increase in lipase production by *Burkholderia cepacia* (Rathi et al. 2001). In many fermentation processes, the presence of metal ions may be inescapable and certain metal ions have been reported to function as enhancers of enzyme production. Metallic

Table 2 Effect of nitrogen source, amino acids, and metal ions on the mycelial growth and lipase production of *A. terreus*[a]

Different sources	Biomass (g/50 ml)	Lipase activity (U/ml))	Final pH
Nitrogen sources[b]			
Peptone	0.1497±0.007 e	725±106.06 d	3.9
Tryptone	0.1489±0.013 e	550±70.71 e	4.5
Gelatin	0.1067±0.003 f	125±35.35 f	3.9
Yeast extract	0.1931±0.009 d	800±70.71 d	4.7
Beef extract	0.2535±0.025 c	1075±106.06 cd	5.1
Ammonium persulphate	0.9834±0.033 a	2575±35.35 b	5.3
Ammonium nitrate	0.1024±0.012 f	475±176.77 e	4.8
Ammonium sulphate	0.2733±0.007 bc	1225±247.48 bc	5.5
Ammonium chloride	0.3751±0.010 b	1625±106.06 bc	4.1
Sodium nitrate	0.2693±0.012 c	1125±176.77 c	5.6
Urea	0.1107±0.009 f	450±0.00 e	4.9
Amino acids (1 mM)			
Alanine	0.4497±0.003 ab	675±35.35 de	6.2
Proline	0.7745±0.007 ab	1200±70.71 bc	6.0
Valine	0.5165±0.008 ab	525±35.35 e	6.4
Aspartic acid	0.4166±0.002 ab	1000±70.71 cd	4.8
Methionine	0.5096±0.001 ab	1050±70.71 cd	6.2
Glutamate	0.3406±0.001 b	925±35.35 cd	6.3
L-lycine	0.3899±0.002 b	975±35.35 cd	6.3
Cysteine	0.3847±0.014 b	950±70.71 cd	6.3
Histidine	0.4171±0.013 ab	800±0.00 d	6.5
Phenyl alanine	0.3003±0.019 b	1050±70.71 cd	6.1
Isoleucine	0.4755±0.011 ab	1225±35.35 bc	6.3
Threonine	0.3313±0.004 b	850±70.71 d	6.2
Tryptophan	0.4703±0.026 ab	950±70.71 d	6.3
Arginine	0.2775±0.010 bc	825±35.35 d	6.1
Leucine	0.3574±0.014 b	1125±35.35 bc	6.4
Glycine	0.3189±0.012 b	1175±35.35 bc	6.2
Serine	0.4005±0.005 b	750±70.71 d	6.3
Metal ions (1 mM)			
Zn^{++}	0.0956±0.003 f	725±35.35 d	4.3
K^{+}	0.8803±0.001 a	275±35.35 f	5.9
Ag^{++}	0.0755±0.005 f	1050±70.71 cd	3.9
Fe^{++}	0.9201±0.004 a	625±35.35 de	4.5
Mg^{+}	0.9615±0.007 a	2775±35.35 a	5.7
Cu^{++}	0.0847±0.001 a	600±0.00 de	4.1
Mn^{+}	0.8656±0.008 a	475±35.35 e	7.2
Ca^{+}	0.8695±0.003 a	2500±50.00 b	5.3
Hg^{+}	0.0593±0.001 g	625±35.35 de	3.5
EDTA	0.050450.001 g	450±70.71 e	5.2

Data are presented as the mean ± SD of replicates (n=3). Values in the same column followed by different lower-case letters are significantly different between treatments and the control at $P \leq 0.05$

[a]The LSSC experiments were performed for 4 days at 30 °C

[b]Different nitrogen sources were added to the basal medium at concentrations equivalent to 1 % (w/v)

ions are believed to influence enzyme production, with those like Ca^{2+} possibly forming complexes with ionized fatty acids, thereby altering their solubility and behavior at oil–water interfaces and consequently inhibiting enzyme synthesis (Tan et al. (2004). However, Ca^{2+} and Mg^{2+} do not appear to have inhibited lipase production in *A. terreus*.

Effect of vitamins

The effect of the four vitamins (vitamin C, riboflavin, folic acid and vitamin E) tested here on mycelial growth and lipolytic activity of *A. terreus* are shown in Table 3. In general, the presence of vitamins affects the biosynthetic rate of metabolites. However, the influence of vitamins on

Table 3 Effect of various antioxidant vitamins on lipase and mycelial biomass production

Vitamins	Concentration (mg/100 ml)	Lipase activity (U/ml)	Biomass (g/ 50 ml)
Vitamin C	10	900±282.84 cd	0.235±0.003 e
	20	925±35.35 cd	0.783±0.029 b
	30	925±35.35 cd	0.134±0.016 f
	40	1325±318.19 b	0.148±0.008 f
	50	1100±0.0 c	0.223±0.0018 e
Riboflavin	10	525±35.35 f	0.210±0 .0092 e
	20	525±35.35 f	0.692±0.0078 b
	30	625±35.35 e	0.347±0.012 d
	40	675±35.35 e	0.883±0.001 a
	50	950±70.71 cd	0.931±0.004 a
Folic acid	10	125±35.35 g	0.356±0.001 d
	20	700±70.71 de	0.385±0.003 d
	30	800±0.0 d	0.475±0.007 cd
	40	825±35.35 d	0.563±0.008 c
	50	1025±35.35 c	0.247±0.019 e
Vitamin E	10	900±35.35 d	0.536±0.014 c
	20	925±35.35 cd	0.521±0.013 c
	30	975±35.35 cd	0.425±0.021 cd
	40	975±35.35 cd	0.143±0.026 f
	50	975±35.35 cd	0.367±0.014 d
[a]Control	-	1675±35.35 a	0.039±0.013 b

Data are presented as the mean ± SD of replicates (n=3). Values in the same column followed by different lower-case letters are significantly different between treatments and the control at $P \leq 0.05$

The LSSC fermentation setups were performed for 96 h at 30 °C

[a] Control refers to fermentation broth medium with MoC added (10 % w/v) and devoid of vitamins

biomass and lipase production have remained unevaluated until this study. We examined the effect of adding these four vitamins to the fermentation medium at concentrations ranging from 10 to 50 mg/100 ml. Compared to the other three vitamins tested, vitamin C at 40 mg/100 ml produce significantly more lipase; however, lipase activity was lower in the presence of vitamin C than in the control. These results suggest that the supply of vitamins are not an absolute demand for the lipase production and growth by *A. terreus*. Similar observations have been described in *Volvariella esculenta* (Jonathan et al. 2004) and *V. speciosa* (Fasidi and Akwakwa 1996).

Effect of mixed substrates on lipase production

The effect of combined agro-wastes on lipase production were studied, and MoC:NoC at a ratio of 9:1 and MoC:GnoC at a ratio of 5:5 were found to give the maximum specific activity (Table 4). All combinations had positive

Table 4 Effect of combined agro-wastes on enzyme production

Combination of agro-wastes	Ratio	Specific activity (U/mg)
MoC:GnoC	1:9	342.27±35.35 cd
	3:7	349.16±35.35 c
	5:5	370.18±70.71 b
	7:3	351.24±70.71 c
	9:1	339.62±106.06 cd
MoC:NoC	1:9	348.49±70.71 c
	3:7	341.69±35.35 cd
	5:5	365.98±70.71 bc
	7:3	329.98±35.35 ed
	9:1	390.89±70.71 a
GnoC:NoC	1:9	340.49±70.71 cd
	3:7	316.38±70.71 d
	5:5	351.61±35.35 c
	7:3	348.86±106.06 c
	9:1	346.43±35.35 c
Only MoC[a] (10 %)	-	281.90±35.35 e

Data are presented as the mean ± SD of replicates (n=3). Values in the same column followed by different lower-case letters are significantly different between treatments and the control at $P \leq 0.05$

The SSF setups were performed as per the above combination for a period of 96 h at 30 °C

[a] SSF medium with MoC (10 %w/v) served as the control

effects and resulted in the higher lipase production. This finding can lead to industries developing specific combinations for optimum lipase production. Combined substrates may enhance lipase production and many researchers have also applied different substrate combinations (Rapp 1995; Dalmau et al. 2000). In our study, we tested combinations of agro-wastes and found that these combinations support the biosynthesis of lipase; this is the first such study using *A. terreus*.

Effect of inoculm size

In order to determine the effect of inoculum size on lipase production, we tested inoculum in the range of 2–10 % (w/v) and measure the effect by making spore counts with a hemocytometer. Increased enzyme production was observed with increasing in inoculum size, with maximum enzyme activity at 96 h with 6 % (w/v) inoculum (Fig. 5). Further increases in inoculum size resulted in decreased enzyme synthesis. However, compared to the control, there was no significant effect of the size of the inoculums on lipolytic activity. In their study, Elibol and Ozer (2000) observed that no morphological variations occurred once the spore had germinated and grown on the support material. At a suitable inoculum size, the nutrient and oxygen levels allow for sufficient growth of fungus and therefore may

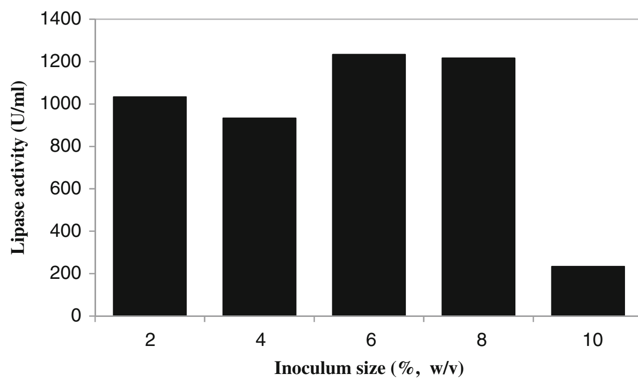

Fig. 5 Effect of inoculum size on lipase production by *A. terreus* cultured in a solid state fermentation system (SSF) for 4 days at 30 °C

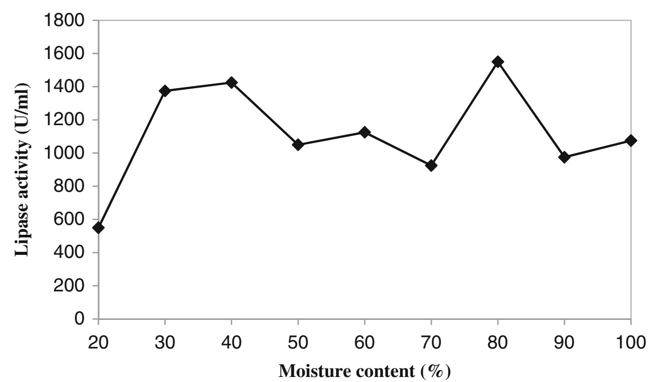

Fig. 6 Effect of initial moisture content on lipase production by *A. terreus* cultured in a SSF system for 4 days at 30 °C

enhance the lipase production. If the inoculum size is too small, insufficient biomass will lead to reduced levels of secreted lipase. A too high inoculum size can cause a lack of oxygen and nutrient depletion in the culture media, resulting in poor product yield (Mudgett 1986). Further increases in inoculum size can result in decreased enzyme synthesis, probably due to nutrient limitation.

Effect of moisture content

Only a very limited amount of water is present in the SSF system and thus an optimum moisture content is imperative as it determines the productivity of a SSF process. Moisture content in a SSF system can vary due to evaporation of the existing water through metabolic heat evolution, water consumption and liberation through fungal metabolism, as well as due to environmental factors. The moisture content in the substrate also depends on the type of microorganisms used in the SSF. At the same time, the amount of moisture content also varies depending on the water-binding characteristic of the substrate. The water content of MoC was adjusted to between 20–100 % in order to examine the effect of moisture content of the substrate on lipase production . The optimum lipase production was observed at 80 %, indicating that the water-binding capacity of MoC was low (Fig. 6). Although these results do not indicate the actual moisture content in the system, they do give an indication of the initial water content required to achieve maximum lipase production and fungal growth. Considering the water loss and the water both consumed and produced during the fermentation, the actual water required by the system is normally not known since water exists in a complex form within the solid matrix on a thin layer either absorbed to the surface of the substrate particle or less tightly bound within the capillary region of the solid. The water that does exists or which is absorbed within the solid matrix is important for fungal growth on the substrate as it will allow an efficient oxygen transport process. The results reported by Pau and Omar (2004) are in accordance with our findings for moisture content.

Effect of growth hormones

In order to determine the effect of growth hormones [gibberellic acid, kinetin,6-benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D)] on lipase production, we supplemented fermentation medium with various growth hormones at the concentration of 0.025 % (w/w) after the medium had been autoclaved. Maximum lipase activity was obtained with BAP (Fig. 7), but all growth regulators produced reduced activity in comparison to control and did not support in any enhancement of lipase biosynthesis. Negi and Banerjee (2010) also studied the effects of growth hormones on amylase and protease production and concluded that indole-acetic acid (IAA) and indole-3-butyric acid (IBA) stimulated the production of both enzymes. Naphthalene-acetic acid (NAA) and 2,4-D enhanced protease production but reduced amylase production. IAA was more effective than IBA in enhancing the production of amylase and protease, which does not support our findings in general.

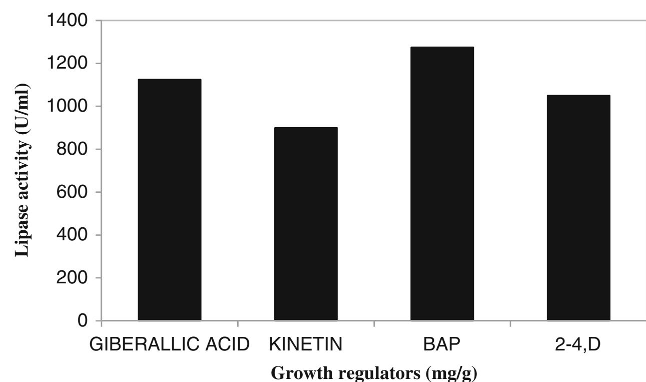

Fig. 7 Effect of different growth regulators on lipase production by *A. terreus* cultured in a SSF system for 4 days at 30 °C. *BAP* 6-Benzylaminopurine, *2,4-D* 2,4-dichlorophenoxyacetic acid

Conclusion

Through modification of media components, we achieved optimization of the fermentation parameters within a short fermentation time; in addition agro-waste combinations enhanced enzyme production. The utilization of low-cost agro-wastes (MoC) may lead to less expensive components being used in the culture medium and an overall reduction in the cost of lipase biosynthesis. Inexpensive medium that allows a better recovery and purification of lipase may be promising for industrial exploitation. Therefore, with the increase in yield, productivity and simultaneous cost reduction, the production of lipase by *A. terreus* on an industrial scale can be regarded as both possible and economically attractive.

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