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*Full Length Article*

# Extracellular Lipase Production by *Aspergillus nidulans* (MBL-S-6) under Submerged Fermentation

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## Abstract

In the present studies, a number of putrefying food items were used in order to isolate various inhabiting fungi. Isolates from cooked masala rice were further screened for production of lipase and a hyper producer was identified and codified as *Aspergillus nidulans* MBL-S-6. The isolate was subjected to submerged fermentation using different cultural conditions for enhanced production of extracellular lipases. Results were evaluated both in terms of specific and enzyme activity. Rice bran was optimized as the best additive to the basal medium with enzyme activity of 33.3±0.00 U/mL/min. A more or less gradual increment in enzyme and specific activity was observed when experiments were conducted by applying differential levels of time course, spore inoculum size, volume of fermentation medium, pH, temperature, carbon additive, etc., were applied. Maximum enzyme activity was recorded after 72 h of incubation with inoculum level of 2 mL (*i.e.,* 27.0±2.77 U/mL/min). Forty five milliliter of fermentation medium at pH of 7.00 was optimized for enhanced enzyme production *i.e.*, 102±2.77 U/mL/min, when provided with 1% starch as additional carbon source to the diluent. A temperature of 40°C proved ideal for maximal lipase production. © 2014 Friends Science Publishers

**Keywords:** Lipase; Biosynthesis; *Aspergillus nidulans;* Eco-cultural optimization; Fermentation

## Introduction

Lipases, triacyl glycerol acyl-hydrolases (E.C. 3.1.1.3), are enzymes that are inherently responsible for catalyzing the hydrolysis of triglycerides to di- and mon-oacylglycerides. These are characterized by working in oil-water interface and their end products are simple acylglycerols, free fatty acid and glycerol. Living system utilize lipases for the esterification, transesterification and resolutions of chiral substrates. (Fickers *et al.,* 2011; Padilha *et al.,* 2012). Most of the chemical reactions are catalyzed by lipases in both aqueous and non-aqueous media due to the use of different substrates, organic solvent and their ability to survive at high temperature and pH (Saxena *et al*., 2003). Lipases are commercially being applied in paper and agrochemical industry (Hiol *et al.,* 2000; Brink and de Vries, 2011), in food Industry for flavor enhancement, in cheese ripening, and ester production (Liu *et al.,* 2012; Couto and Sanoromana, 2006), in pharmaceuticals, cosmetics and in oil industry (Rekha *et al.,* 2012).

Lipases are ubiquitous enzymes and are reported from different sources like plants, animals, fungi, bacteria and yeast (Saxena *et al.,* 2003; Salihu *et al.,* 2012; Houde *et al*., 2004). Amongst different living sources, fungi have gained significant attention as well as importance because these are considered to be the cheapest source of lipase biosynthesis (Sharma *et al.,* 2001; Iftikhar *et al*., 2012). Species belonging to *Aspergilli, Rhizopus, Mucor and Penicillia* are considered to be good producer of commercially important enzymes (Mahadik *et al*., 2002; Rekha *et al.,* 2012).

There are a number of well-defined fermentation techniques. However, submerged fermentation is preferable because cultural variables can easily be controlled under submerged fermentation (Li and Zong, 2010). Furthermore, different agricultural by-products used as additive to the fermentation medium are being employed for hyper-production of lipases (Fadiloglu and Erkmen, 2002).

Pakistan is an agricultural country with a number of policy options for transformational changes toward industrial economy. Enzyme technology has grown enormously during recent past. At the moment, almost all its needs are being met by importing enzymes. Geographic location of Pakistan is suitable for the sustenance of organisms having inherent capacity to survive under environmentally harsh conditions. These types of organisms are suitable for the production of thermostable and thermophilic enzymes (Niaz, 2003; Ahmad and Butt, 2013). The baseline data generated through this piece of work will not only go a long way in process optimization of production but also pave the way for characterization and more comprehensive molecular studies on lipases produced by *A. nidulans* and their subsequent recommendation for industrial employment that can contribute in the economic uplift of the country. The objective of the present piece of work was to optimize the cultural conditions in order to maximize the extracellular lipase production.

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## Material and Methods

### Microorganism

An unidentified fungal culture was picked from the culture collection of Laboratory of Biotechnology, Department of Botany, Govt. College University, Faisalabad. Culture was maintained on the 4% Potato dextrose agar (PDA) slants throughout the study period. Microscope (MEIJI Model: ML2100) was calibrated and different micrometric and micro-morphological measurements were made for the identification of hyper producer by using standard monographs (Mirza and Hussain, 1966; Rizvi, 1966; Qureshi, 1966; Hussain *et al.,* 1968; Hussain and Ahmed, 1971; Ghaffar and Abbas 1972; Ahmed *et al*., 1997) and web sources (Doctorfungus. www.doctorfungus.org).

### Spore Inoculum

Spore inoculum was prepared by addition of 10 mL sterilized distilled water in 5-7 days old culture. Spores were scratched by means of inoculating needle and aseptically transferred to fermentation medium as reported by (Iftikhar *et al.,* 2012).

### Fermentation Technique

Twenty five milliliter of fermentation medium containing g/100mL glucose (1 g), peptone (2 g), yeast extract (0.5 g) and sodium chloride (0.5 g) was transferred to each 250 mL erlenmeyer flask plugged with cotton wool. The flasks were sterilized in autoclave at 121$^{o}$C, 15 lb/in$^{2}$ for 15 min and cooled at room temperature. One mL of inoculum was aseptically transferred to each flask. The flasks were placed in orbital shaking incubator for incubation at 30$^{o}$C with shaking speed of 200 rpm. After 72 h of incubation the contents of flask were used for the estimation of total protein and enzyme. The data was collected from the experiments performed in triplicate after following Iftikhar *et al.* (2012).

### Titrimetric Assay

Lipase activity in the medium was determined titrimetrically on the basis of olive oil hydrolysis as described by Kempka *et al.* (2008). One enzyme unit is the amount of enzyme, which releases one micromole fatty acid per minute under specified assay conditions and expressed as U/mL/min.

### Protein Estimation

Protein micro-assay was performed after following Bradford (1976). Bovine serum albumin (BSA) was used to draw standard curve.

### Specific Activity

It was calculated by dividing enzyme activity (U/mL/min) with total protein produced (U/mg).

### Statistical Data Analysis

Co-stat software was used for experimental data analysis and graphs were prepared by plotting the data in MS excel (2007).

## Results

### Identification of Fungus

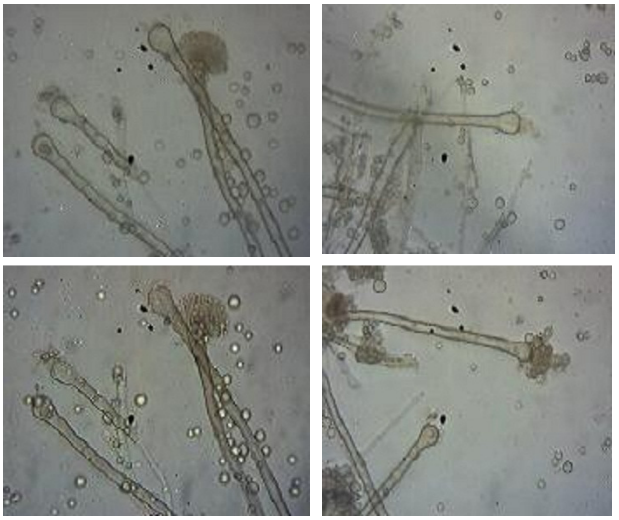
Micrometry and micrography was done for technical description of the unknown culture (Fig. 1). The hyper-producer was identified as *Aspergillus nidulans* and assigned the code MBL-S-6.

### Optimization of Cultural Conditions

**Selection of agro-industrial by-product as additive to the fermentation medium:** Different agro industrial by-products like 1% (w/v) wheat bran (WB), rice bran (RB), brassica bran (BB), almond meal (AM) and mustard meal (MM) (w/v) were used as additive under submerged fermentation using *A. nidulans* (MBL-S-6) and the results in terms of extracellular lipase production and specific activity were compared. Rice bran was proved to be hyper-producer among all these substrates with maximum extracellular lipase units, *i.e.,* (33.0±0.04 U/mL/min). At this point specific activity of lipase was also maximum (24.67±0.06 U/mg). On the other hand wheat bran as additive showed the lowest value of extracellular lipase units (11.0±2.80 U/mL/min). Other substrates showed intermediate values as presented in (Fig. 2). Therefore, rice bran was optimized as the best substrate and further studies were conducted with this substrate.

### Size of Inoculum

Different inoculum size, *i.e.,* 0.5, 1, 1.5, 2, 2.5, 3 mL were employed to submerged fermentation and then compared their results in order to determine their effects on lipase production by *A. nidulans* (MBL-S-6). Among all these applications, the batch having 2 mL inoculum gave maximum extracellular lipase activity, i.e., (25±0.02 U/mL/min) at 72 h. Specific activity of lipase was also found to be maximum*, i.e.,* (15.25 U/mg) (Fig. 3) It was also noted that 0.5 mL concentration of inoculum gave lowest values of lipase activity i.e., (8.0±0.01 U/mL/min) with better specific activity (10.3±0.02 U/mg). While other inoculum concentrations as 1, 1.5, 2.5 and 3 mL showed intermediate values of lipase production and specific activity. Therefore, 2 mL inoculum size was optimized for further studies.



**Kingdom: Fungi**
**Phylum: Ascomycota**
**Class: Eurotiomycetes**
**Order: Eurotiales**
**Family: Trichocomaceae**
**Genus: *Aspergillus***
**Species: *nidulans***

**Fig. 1:** Classification and microscopic images of *Aspergillus nidulans* (MBL-S-6)

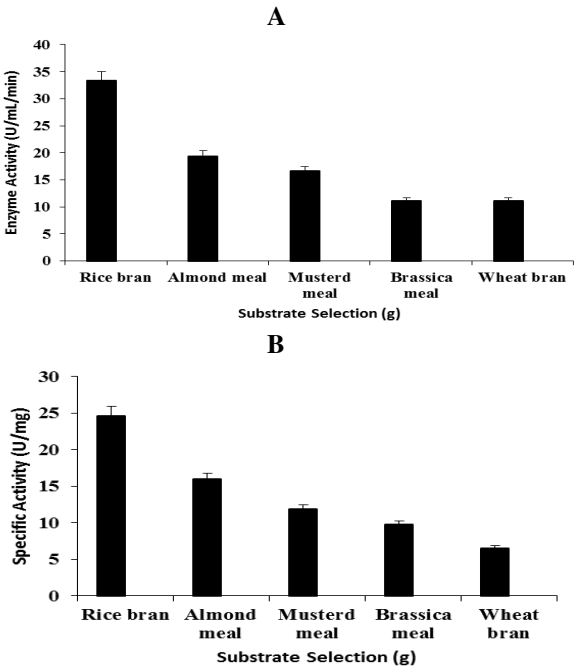



**Fig. 2:** Comparison of different agro industrial byproducts as additives for extracellular lipase production and specific activity by *A. nidulans* (MBL-S-6) under submerged fermentation conditions A) Extracellular Lipases B) Specific activity

**Rate of Fermentation**

Time course plays important role towards enzyme production. Nine incubation time courses from 0 h to 192 h were evaluated with interval of 24 h, in order to study the lipolytic potential of *A. nidulans* (MBL-S-6). Apart from lipase units, specific activity by *A. nidulans* (MBL-S-6) was also determined at each time course interval. The results are presented in Fig. 4. At 72 h of incubation the extracellular lipase showed its highest activity (27.0±2.77U/mL/min). At this point, the specific activity was also maximum *i.e.,* (27.42 ±0 .03U/mg). It was noted that there was a constant decrease in specific activity values after 72 h of incubation period. Therefore, 72 h was selected as optimized incubation period for further studies.

**Volume of Fermentation Medium**

Submerged fermentation was performed by taking varying volume *i.e.*, 15 mL, 25 mL, 35 mL, 45 mL and 55 mL of 1% w/v rice bran with optimized conditions of the previous experimentation for determining their effect on lipase production and specific activity by *A. nidulans* (MBL-S-6). Enzyme and specific activities of lipase are presented in Fig. 5. After previously optimized incubation period, it was observed that 45 mL of fermentation medium gave maximum extracellular lipase activity *i.e.,* (44±2.77 U/mL/min) with specific activity (48.1±0.07 U/mg). Minimum values of these parameters were recorded at 15 mL of fermentation medium. Other quantities of fermentation medium showed intermediate values as shown in Fig. 5. Therefore, 45 mL volume of fermentation medium was selected for further studies.

**Initial pH of Fermentation Medium**

A range of pH, i.e., 5-9 of fermentation medium was applied in order to check its effect on enzyme production. The data pertaining to lipase production and specific activity of *A. nidulans* (MBL-S-6) is presented in Fig. 6. After 72 h of incubation maximum extracellular enzyme activity i.e., (91.7±0.02 U/mL/min) was achieved at pH 7 with maximum specific lipase activity (127.7±0.09 U/mg). From this optimal point the activity dropped appreciably on both sides of pH range that showed a neutrophilic behavior of the fungus.

**Incubation Temperature**

Incubation temperature range from 25 to 45°C was applied to triplicate batches of submerged fermenting medium containing 1% w/v rice bran with inter-application difference of 5°C. The data was compared for the determination of their effect on lipase production and specific activity by *A. nidulans* (MBL-S-6) (Fig. 7). It was observed that the maximum extracellular lipase activity

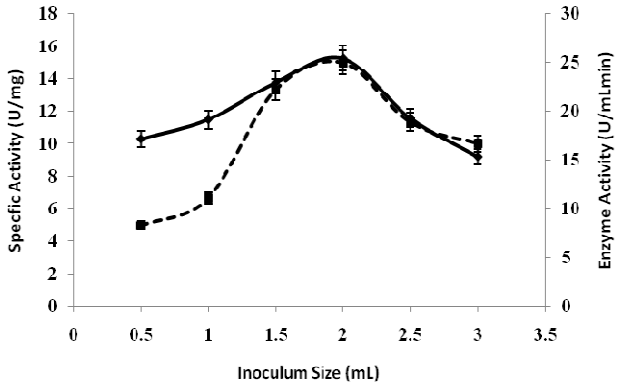



**Fig. 3:** Comparison of different inoculum size on extracellular lipase production of *A. nidulans* (MBL-S-6) under submerged fermentation (*Legend:* ♦= *Specific Activity*, ■= *Enzyme Units*)

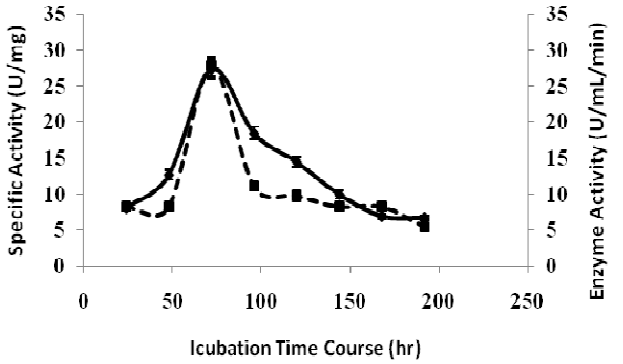



**Fig. 4:** Comparison of different incubation period on the production potential of *A. nidulans* (MBL-S-6) for extracellular lipases under submerged fermentation conditions (*Legend*: ♦= *Specific Activity*, ■ = *Enzyme units*)

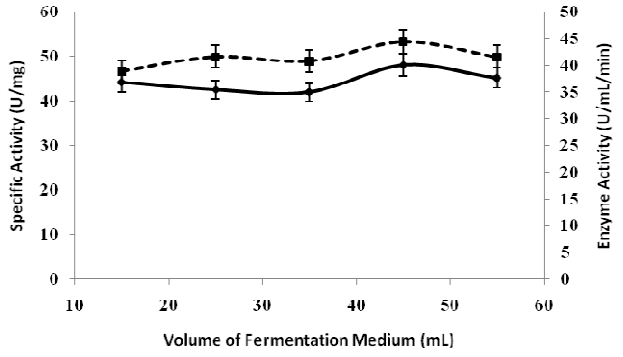



**Fig. 5:** Comparison of different volume of fermentation medium on extracellular lipase production of *A. nidulans* (MBL-S-6) under submerged fermentation. (*Legend*: ♦ *Specific Activity*, ■= *Enzyme Units*)

(86±5.57 U/mL/min) with specific activity (47.8±0.07 U/mg) was recorded at 40°C after 72 h of incubation period at pH 7 (Fig. 4). Minimum enzyme units (49 ±2.70

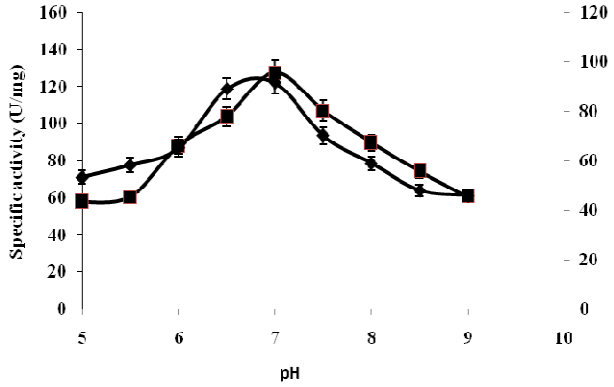



**Fig. 6:** Relationship of pH and extracellular lipase production by *A. nidulans* (MBL-S-6) under submerged fermentation. (*Legend:* ♦= *Specific Activity*, ■= *Enzyme Units)*

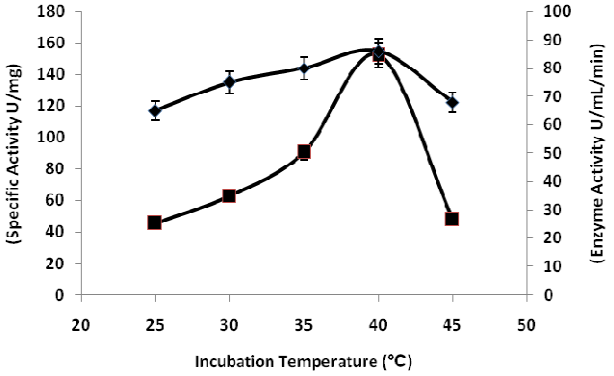



**Fig. 7:** Comparison of different incubation temperatures on extracellular lipase production of *A. nidulans* (MBL-S-6) under submerged fermentation (*Legend*: ♦=*Specific Activity*, ■= *Enzyme Units*)

U/mL/min) and specific activity (37.6±0.06 U/mg) was recorded at 35°C. On the basis of the data obtained out of this experiment, 40°C was selected as optimum incubation temperature for further studies.

**Different Additives to Fermenting Medium**

The effect of different carbon additives *i.e.,* sucrose, lactose, starch, glucose and dextrose and nitrogen additives i.e., casein, yeast extract, peptone, nutrient broth, urea, ammonium sulfate, ammonium nitrate, ammonium chloride and ammonium molybdate each @ 1% (w/v). The results were compared for their effect on lipase production and for specific activity (Fig. 8). The starch was considered as additive that results in maximum extracellular enzyme *i.e.,* (102 ±2.77 U/mL/min) with maximum specific activity i.e., (62.17±0.00 U/mg). It was further observed that sucrose gave minimum lipase units (69±2.67U/mL/min) as well as specific activity (52.27±0.00 U/mg). Therefore, starch was selected as optimized additional carbon source for further studies.

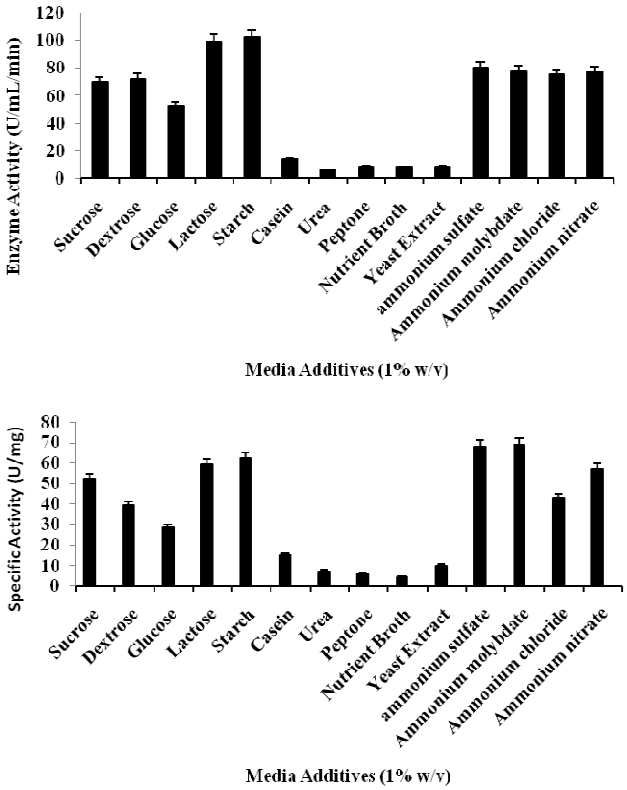



**Fig. 8:** Effect of additives to basal fermentation medium on A) Enzyme activity B) Specific activity of lipases by *A. nidulans* (MBL-S-6) under submerged fermentation

## Discussion

Lipases, triacylglycerol acyl hydrolases (E.C. 3.1.1.3) are involved in the hydrolysis of triacylglycerol to free fatty acids and glycerol (Sharma *et al.,* 2001*;* Fickers *et al.,* 2011), to esterification, transesterification and aminolysis in organic solvents (Joseph *et al.,* 2008). Lipases have the capability to mediate reactions in organic solvents as they possess low water activity that tends to precede the reacting molecules for synthesis rather towards ester hydrolysis (Gumel *et al*., 2011).

An unknown wild strain MBL-S-6 was picked and exploited in order to check its lipolytic potential through submerged fermentation technique. Different optimization parameters for enzyme productions like pH, temperature, inoculum size and type, different substrate under varying concentration, time course intervals for incubation were studied during present work.

Cost effective medium of enzyme production is always a cherished goal during process optimization of enzyme production (Mohseni *et al.,* 2012). Fungi possess differential lipase production potential towards different agro-wastes (Fadiloglu and Erkmen*.,* 2002). Therefore, a variety of agro-industrial waste additives (1%) like almond meal, mustard meal, brassica meal, rice bran and wheat bran under varying concentration were applied for optimizing the best basal medium of lipase production under submerged fermentation. Rice bran additive gave the maximum enzyme units and specific activity for lipases. Earlier, Rao *et al*. (1993) and Mohseni *et al*. (2012) reported that rice bran is the preferential substrate for lipase biosynthesis among the fiber rich substrates. Furthermore, rice bran oil had been reported to trigger lipase hyper production in *Bacillus* THL027 (Dharmsthiti and Luchai, 1999). From the present study and previous reports it is evident that although rice bran is a fiber rich substrate yet any of its active ingredients possess a triggering role for hyper-production of lipases. For which more comprehensive studies are needed. The elucidation of any triggering factor of lipase production in rice bran may give a breakthrough in defining an efficient commercial lipase production. There are some astonishing reports that fungi utilize both lipid and non-lipid substrates for enhanced production of lipases such as almond meal (Iftikhar *et al.,* 2012), wheat bran (Edwinoliver *et al.,* 2010; Kumar *et al.,* 2011; Nagar *et al.,* 2011).

Incubation time course also play a pivotal role in lipase biosynthesis. During present work 72 h of incubation with rice bran @ 1% (w/v) under submerged fermentation was optimized for enhanced lipase production by *A. nidulans* (MBL-S-6). Different workers reported differential incubation time course for maximum lipase production for different fungal species. It had also been reported that lipase activity found to be maximum after 96 h in *A. terreus* by (Gulati *et al*., 1999)*,* in *Rhizopus arrhizus* by (Yang *et al*., 2005), in *Fusarium solani* FS1 (Maia *et al.,* 2001*).* After 24 h of incubation period in *R. arrhizus* and *A. niger* by (Mahadik *et al.,* 2002) showed maximum lipase activity. The studies are in accordance with Edwinoliver *et al.,* 2010 who reported similar incubation time for maximum lipase production by *A. niger* MTCC 2594, (Sun and Xu*,* 2008) for *R. chinensis* and Kumar *et al.* (2011) for *Penicillium chrysogenum.*

There was a linear increase in lipase production and its specific activity with increasing spore inoculum size from 0.5 to 2 mL. Beyond which lipase production decreased under previously optimized cultural conditions. The possible explanation to decrease in enzyme biosynthesis at elevate spore inoculation may be due to the presence of large mycelial mass that used the substrate in large quantities and leaving non-lipid part of nutrient for the sustenance of its life. Iftikhar *et al.* (2012) also reported similar inoculum size for increased lipase production. Our notion of decrease in lipase production is further confirmed by looking at a sharp decrease of specific activity of lipases from 2 to 3 mL inoculum size.

During present study, different volume of fermentation medium, from 15-55 mL were employed to previously optimize eco-cultural parameters. A media volume of 45 mL gave best lipase biosynthesis and their specific activity. Our results are slightly different from the findings of Iftikhar

*et al.* (2012), who reported that a volume of 50 mL is ideal for lipases production using *A. niger.*

Production potential of *A. nidulans* (MBL-S-6) in terms of pH of the medium for lipase biosynthesis revealed that pH 7 was optimal that reflects its neutrophilic behavior. This pH is extremely easy to maintain during mass production and it can be inferred that this fungus can be a good candidate for lipase production, if other parameters and characteristics of its lipases qualify for industrial employment. Kamini *et al*. (2000) reported similar results for *Cryptococcus* sp. S-2, Kumar *et al.,* (2011) for *P. chrysogenum* and Gupta *et al*., (2007) for *Burkholderia multivorans*. However, other fungi responded well in other pH optima like *P. simplicissimum* at pH 5 (Gutarra *et al.,* 2009), *R. chinensis* at 6.5 (Sun and Xu, 2008), *R. oryzae* at 7.5 (Yuzbashev *et al.,* 2012) and in *Antrodia cinnamomea* BCRC 35396 at 8 (Shu *et al.,* 2006) and in *Burkholderia* sp. C20 at pH 9 (Liu *et al.,* 2012).

During the study for finding the optimal temperature of lipase production, maximum enzyme production was obtained at 40°C. This temperature was found to be optimal for most of the fungal species and for further lipase production, *e.g.,* Falony *et al*. (2006) reported this for *A. niger* (J-1); Kempka *et al.* (2008) for *P. verrucosum* and Shu *et al.* (2006) for *A. cinnamome* BCRC 35396. However, there had been reports of a range of temperature optima for lipase production, e.g., 30°C in *P. chrysogenum* (Ramani *et al.,* 2010*)*; 37°C in *A. terreus* (Gulati *et al.,*1999), between 30-40°C in *R. oryzae* (Yuzbashev *et al.,* 2012) and 40°C in *Sporidiobolus ruberrimus* (Oliveira *et al.,* 2012). Our finding reveals *A. nidulans* to be slightly thermophilic and a good candidate for industrial employment. However, more comprehensive studies regarding characterization of lipases of this fungus are required before making final recommendation of its commercial application.

During the final phase of eco-cultural optimization, different carbon and nitrogen additives were added to the fermenting medium. Maximum lipase and their specific activities were obtained when starch was added @ 1% (w/v). Earlier, Pokorny *et al*., (1994) reported an increase in lipase production by using starch as additive. The probable production enhancement might be that the consortia of amylases and lipases act in a synergistic manner in amylolytic degradation of starch into glucose monomers which upon further splitting yield glycerates that triggers lipid metabolism within thallus.

Conclusively we are of the opinion that *A. nidulans* (MBL-S-6) is a good source for lipase. This data can be taken as base-line study and comprehensive studies in terms of thermophilicity, thermostability, reaction kinetics and thermodynamics are needed for their industrial employment.

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