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IN SILICO PHYLOGENETIC ANALYSIS OF FUNGAL LIPASE GENES AND HARNESSING THE INHERENT POTENTIAL OF *ASPERGILLUS NIGER* IBP2013 FOR EXTRACELLULAR TRIGLYCEROL ACYL-HYDROLASE PRODUCTION UNDER SOLID STATE FERMENTATION

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Abstract

Mesophilic fungi have evolved inherently thermostable/thermophilic enzyme production potentials over the years. To explore this potential, fungal colonies were isolated from rotten food items and screened, based on their extracellular triglycerol acyl-hydrolase (lipase) potential over lipid-rich agro-wastes. Hyper-lipase producing fungus was identified by comparing with standard fungal morpho-graphs as *Aspergillus niger* (*A. niger*) IBP2013. In present study, coconut meal was found a potential medium for fungus to produce higher extracellular lipase enzyme (lipase activity (la), 23.7±0.25 U/mL min^{-1}; lipase specific activity (lsa), 97.4 U/mg) under solid state fermentation. This inherent potential of the fungus was enhanced up to 41.1±0.4 U/mL min^{-1} (la) and 169.5 U/mg (lsa) by eco-cultural optimization like substrate (coconut meal) and adequate concentrations (10g), inoculum level (1mL), incubation period (72h), temperature (40º C) and pH (7). Moreover, the concentrations of organic (peptone, 1% w/v) and inorganic nitrogen (ammonium acetate, 1% w/v) and carbon (lactose, 0.8% w/v) supplements were found essential for optimal lipase production. Three known fungal genes (*Lip1, Lip2* and *Lip3*) were utilized for *in silico* phylogenetic analysis. Hence, *A. niger* IBP2013 with optimized cultural conditions could be potential candidate for lipase production at commercial scale.

Key words: *Aspergillus niger*, Ecocultural optimization, Extracellular enzyme, Lipases, Phylogenetic analysis.

Introduction

As biocatalyst, enzymes are the integral part of almost all biological metabolic pathways and commercially being applied in food, pharmaceutical, agrochemicals, paper and detergents (Rekha *et al*., 2012; Ray, 2012; Liu *et al.*, 2012). Different sources such as plants, animals and microbes are available for commercial production of enzymes (Ray, 2012). As compared to animal and plant based sources, microbes are considered economically more feasible due to their easy availability and isolation. They are easy to culture with ability to grow rapidly (Sharma *et al*., 2001).

The commonly known lipases, triglycerol acyl-hydrolases are responsible for most of lipolytic metabolic reactions. These are the major source of smaller units of glycerol along with free fatty acids obtained by catalyzing long chain glycerols. Reactions including acidolysis, alcoholysis, hydrolysis, aminolysis, esterification as well as inter-esterification are catalyzed by lipases (Jaeger & Eggert, 2002; Fickers *et al*., 2011). Commercially, lipases are the third most important group of enzymes after amylases and proteases. (Saxena *et al*., 2003; Metzger & Bornscheuer, 2006).

Fungal species such as *Aspergillus niger*, *A. nidulans*, *Alternaria alternata*, *Rhizopus species*, *Pencillium species*, have been explored to produce lipases (Herrgard *et al*., 2000; Iftikhar *et al*., 2014; Mauti *et al*., 2016; Pandey *et al*., 2016). Among these *A. niger* and *A. nidulans* are considered as important ones by industrialists due to their production and stability parameters. *Aspergillus sp.* has the ability to survive in various environments including oil polluted contaminated soils as well as rotten lipid containing food items. (Cihangir & Sarikaya, 2004; Brooks & Asamundo, 2011; Mauti *et al*., 2016).

It is well known that *A. niger* has gained importance commercially in industries for its metabolites. Different investigations showed that it can be used for the production of organic acids such as gluconic acid and citric acid, as well as extracellular enzymes like lipases (Brooks & Asamundo, 2011; Mauti *et al*., 2016). Various fungal strains differ in their potential for lipase production based on their growth conditions under varied environmental factors (Falony *et al*., 2006). Fungal enzymes can be categorized into two groups i.e. extracellular and intracellular lipases based on their synthetic site. The former ones are more stable than the later ones because of sulfide bonds that show its more suitability for various applications (Cheetham, 1995).

Commercial fungal lipases are being produced through submerged fermentation (SmF) and solid-state fermentation (SSF). SSF fermentation is more efficient as compared to SmF due to its advantages such as low energy demands, superior yield, higher enzyme titer production, easy laboratory management, simple growth media requirement, as well as ease of purification (Pandey *et al.*, 2000; Aguilar *et al*., 2001; Robinson *et al*., 2001; Socole & Vandenbergh, 2003).

For commercial purposes, varieties of enzymes are being utilized that have important roles in industrial economy. Lipases are being frequently employed in variety

of industries like in detergent industry; for removal of fatty stains (Hasan *et al*., 2006), food industries, paper and agrochemicals (Ramarethinam *et al*., 2002), as well as hide and leather industries (Houde *et al*., 2004; Prazeres *et al*., 2006; Romdhane *et al*., 2010). Moreover, in various pharmaceuticals and cosmetics, the use of lipases as major ingredient is well known (Ray, 2012).

Different growth substrates, including the products of agro-industries with different concentrations found critical for lipase production in fungi. Furthermore, the incubation temperature, the rate of fermentation, and inoculum level have roles in modulating lipase activity (Basheer *et al*., 2011; Kumar *et al*., 2011; Iftikhar *et al*., 2014; dos Santos *et al*., 2014). Furthermore, the enhancement of lipase production is also being carried out with different parameters optimization such as pH, additional carbon and nitrogen sources as additive media (Jia *et al*., 2015). These additives along with eco-cultural conditions are considered very important in fungal strain's specific growth and their capability of enhanced production of lipase but the deviation across the limits is very critical for fungal lipase biosynthesis (Iftikhar *et al*., 2015; Basheer *et al*., 2011; Kumar *et al*., 2011).

On the other hand, to pool out the candidate genes from different fungal strains, *in-silico* data mining is also very crucial with higher genetic potential for the production of extra cellular enzymes. It provides a basic and essential tool for the search of new candidate genes among various fungal species having inherent genetic potential for higher valuable enzymes.

The present study is designed to isolate the native fungal strains from different lipid rich environment and to compare their lipolytic potential to identify the hyper lipase producing fungi. In addition, the eco-cultural optimization crucial for maximal extracellular lipase production potential of the fungus under solid state fermentation was also the main objective of the study along with known lipase genes *in-silico* comparison with diverse origin as well as the identification of fungal lineage.

Materials and Methods

Lipolytic fungal strain isolation: Lipid rich food sources were used for the isolation of lipase producing fungal strains. Amongst those fungal species a hyper-producing strain was identified as *Aspergillus niger* by using standard procedure and consultation with the relevant monographs and web sources. Potato dextrose agar (PDA) medium was used to grow this isolated fungal strain. For the plating before the incubation, about ten mL of the autoclaved PDA medium was utilized. The inoculation of the fungal colonies was done as following Sidra *et al*., (2016).

Maintenance of fungal culture: The fungal strains with lipolytic activity were retained from cultures on the PDA (Chopped potato for potatoes infusion, 200.0 g/L; Dextrose (Sugar), 20.0 g/L; 2% Agar; pH = 4.5) medium. The process was repeated for protection, culture purification, and to obtain the maximum growth of these strains.

Preparations of 1% PDA medium: The medium was prepared by adjusting pH at 4.5. The fungal cultures were maintained following Sidra *et al*., (2016).

Screening methods of fungal strains for lipase production

Fermentation technique: Solid state fermentation technique was used for the isolation of fungal strain for lipase production as described by Vaseghi *et al*., (2013).

Slide preparations and identification of fungal isolates: Lactophenol cotton blue (LPCB) in 70% alcohol on clean glass slide carrying fungal hyphae was used to examine the fungi under microscope as done by Abbas *et al*., (2010). The microscope (LABOMED, Model: Lx 400) was used to examine the prepared slides. To distinguish the selected fungus based on studied characters, standard literature along with web sources (myco bank) were used.

Extracellular lipase biosynthesis assay

Lipase assay: After incubation for 72h, the growth medium was centrifuged at 5000 rpm for 15 min and the supernatant was used for extracellular lipase biosynthesis as described by Kempka *et al*., (2008).

Lipase unit: The enzyme required to release one µmole of fatty acid per ml of medium is known as lipase unit (U/mL min^{-1}) and calculated using the formula:

$$\frac{\Delta VXN}{V\,(Samples)} X \frac{1000}{60}$$

where;

$$\Delta V = V2 - V1$$

V1 = NaOH volume used against control flask
V2 = NaOH volume used against experimental flask
N = Normality of NaOH
V (samples) = volume of used enzyme extract

Cultural condition optimization for enzyme biosynthesis: It is based on the following parameters as described below:

Use of different solid substrates for optimum biosynthesis of extracellular lipase: Different solid substrates such as almond meal, brassica meal, coconut meal, seasam meal, rice and wheat bran were used to optimize the biosynthesis of extracellular lipase following Iftikhar *et al*., (2014).

Effect of different substrate additives on biosynthesis of extracellular lipase: The method described by Iftikhar *et al*., (2012) was used to produce substrate based extracellular lipase.

Inoculums sizes variation for optimum extracellular lipase biosynthesis: The method of Gutarra *et al*., (2005) was used to find out the effects of inoculums size on lipase biosynthesis.

pH and temperature regimes variation for optimum extracellular lipase biosynthesis: The method as described by Iftikhar (2015) was used to find out the effects of medium pH and temperature on lipase biosynthesis.

Incubation T-intervals variation for optimum extracellular lipase biosynthesis: Different incubation time intervals *viz.,* 24, 48, 72, 96 and 120 hours on enzyme production ability were employed as reported by Edwinoliver *et al*., (2010).

Effects of additional C-sources on biosynthesis of extracellular lipase: Different sources (1% each) such as dextrose ($C_6H_{12}O_6$), glucose ($C_6H_{12}O_6$), sucrose ($C_{12}H_{22}O_{11}$), lactose ($C_{12}H_{22}O_{11}$), and starch ($C_6H_{10}O_5$) were used as additive for optimal lipase production following the method described by Iftikhar (2014).

Effect of different N-additives on biosynthesis of extracellular lipase: The method as described earlier by Iftikhar (2014) was followed to find out the effects of different N sources on lipase production. Different organic nitrogen sources (1% each) used separately including the yeast extract, nutrient broth, peptone, casein, and urea. Different inorganic N sources used include the ammonium sulfate $(NH_4)_2SO_4$, ammonium chloride (NH_4Cl), ammonium molybedate $(NH_4)_2MoO_4$, ammonium nitrate (NH_4NO_3), as well as ammonium acetate ($NH_4C_2H_3O_2$).

Use of different concentrations of additives in growth medium: Method as described by Lima *et al*., (2003) was used to find out the effects of different concentrations (0, 0.4, 0.8, 1.2 w/v) of additives on lipase production.

Total protein estimation: Bradford (1976) method was employed to find out the concentration of total soluble protein and the values were estimated through putting the data on standard curve drawn using the dilution range from 0.1 to 2.0 grams of Bovine Serum albumin (BSA).

***In-silico,* phylogeny of lipase gene family:** The genes responsible for lipase biosynthesis in various fungal species were obtained from National Center for Biotechnology Information (NCBI' http://www.ncbi.nlm.nih.gov/). These obtained sequences were aligned with CLUSTALW2 online tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and used to construct phylogeny to study the relationship in lipase gene family (LIP family) among different fungal groups.

Results and Discussions

Identification of fungal strain: In present study, the identification of hyper producer of extracellular lipase producing fungus growing was based on related web sources, relevant monographs, and the standard literature. Based on the morphological and reproductive structures observed, the isolated fungus showed the resemblance to *A. niger*. Micrometry and micrography further confirmed that the fungus was *Aspergillus niger* (Tieghem) (Plate 1A-D). The obligate synonym(s) of *A. niger* are *Sterigmatocystis nigra* (Tieghem, 1867) and *Aspergillopsis nigra* (Tiegh.) Speg., (Spegazzini, 1910).

Eco-cultural optimization for biosynthesis of extracellular lipase: The end products of triglycerol catabolism are the glycerol and free fatty acids, is the function of well-known lipases and triglycerol ester hydrolases (Fickers *et al*., 2011). Lipases are being frequently used in different industries as well as it has vast application in different agricultural departments. Among lipases, belonging to microbial origin are being considered more important as compared to plants and animal origin. Lipases of fungal origin are being considered more important due to their easy isolation availability and culturing techniques (Sharma *et al*., 2001; Ray, 2012).

In present study, different fungal colonies were isolated from different sources (bakery products, sweets, cream and kitchen products etc.) to find out their potential for lipase biosynthesis using solid state fermentation (SSF) technique. Among the studied ones, the fungi *A. niger* IBP2013 was found as hyper lipase producer from rotten cream.

Optimization of agro-industrial wastes as growth medium on extracellular lipase biosynthesis: To harness the ability of *A. niger* IBP2013 for lipase production was cultured at varying cultural conditions with different regimes, such as carbon and nitrogen sources, pH, inoculum level, substrate concentration, incubation temperature as well as incubation time interval as media additives through SSFT.

Among different mediums used for fungal growth the maximum extracellular lipase units (23.7 ± 0.25^a U/mL min^{-1}) and specific activity (97.4 U/mg) were obtained from coconut meal as compared with basal media, after 72h of incubation. However, the minimum lipase activity (12.2 ± 0.67^f U/mL min^{-1}) and specific activity (28.5 U/mg) was observed with rice bran. However, the intermediate values of lipase activity and specific activity were found in other substrates as presenting in Fig. 1. Based on these results, coconut meal was selected as best solid growth medium for A. *niger* IBP2013. Though, the coconut meal has already been used as growth medium (Iftikhar *et al*., 2014), but in present study, it was used as solid growth medium that was not in earlier studies, for *A. niger* IBP2013 to find out its potential for the maximum production of lipase. In an earlier study Rekha *et al*., (2012) used the coconut oil as solid media but not coconut meal and obtained useful results. The better production of lipase in coconut meal might be due to its better potential to provide the best solid support for fungal growth in spite of better nutritional source of fungi in comparison with other substrates. However, in earlier studies on almond meal (Iftikhar & Hussain, 2002) and wheat bran (Edwinoliver *et al*., 2010; Nagar *et al*., 2011; Kumar *et al*., 2011; dos Santos *et al*., 2014) found better solid medium for best lipase production and activity.

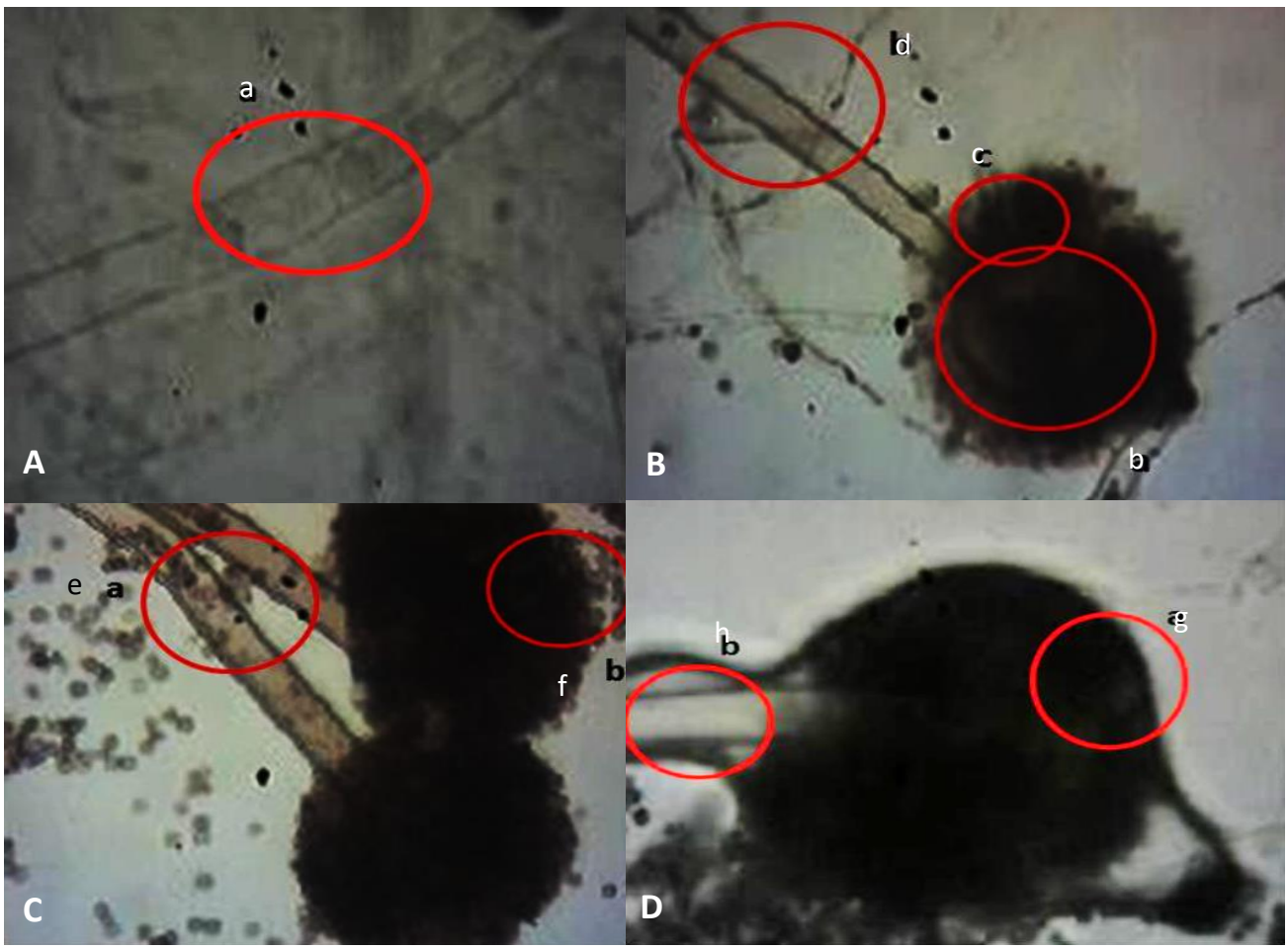

Plate 1. Morphological features of *Aspergillus niger* IBP2013 observed under microscope (labomed) with 100X magnification. A. hyphae, a. septate hyphae (branched); B. dendilone shaped, b. globose vesicles covered with metulae and conidia, c. hyaline to yellowish conidiophore, d. metulae with conidia; C. hyphae dendilone shaped, e. hyaline to yellowish conidiophore, f. spherical shaped conidia; D. conidiophore with conidia, g. dark brown to black conidia, h. hyaline to yellowish conidiophore.

Different incubation time interval and extracellular lipase biosynthesis: It is well known that hyper production of lipase by fungus cultures best correlates with the cultural conditions as reported earlier by Iftikhar *et al*., (2014) and among these optimum time is of prime importance. In present study, from different incubation time intervals (24, 48, 72, 96 and 120h), the highest extracellular lipase units (24.3±0.75 U/mL min^{-1}) and specific activity (99.7 U/mg) were obtained at 72h incubation. However, the minimum lipase activity (13.2±0.35 U/mL min^{-1}) and specific activity (30 U/mg) was observed at 120h of incubation time, but the intermediate values were found at other time intervals (Fig. 2). Thus, the 72h incubation time interval was found best for the *A. niger* IBP2013 and this time interval was used for further studies under solid state fermentation. The findings of present study are in line with some earlier studies as reported by Edwinoliver *et al*., (2010) and Mukhtar *et al*., (2015). They reported that this might be due to less enzyme production at early incubation period but at late incubation period this decrease might be due to depletion of nutrients, less moisture availability for growth as well as the accumulation of noxious products. The time interval of 30h and 48h has also been found best for maximum lipase production as reported by Iftikhar *et al*., (2014). Furthermore, in another study the time interval of 96h was found best for maximum lipase production as reported by Thota *et al*., (2012).

Media volume and extracellular lipase biosynthesis: To optimize another cultural condition, various substrate concentrations were utilized in SSFT in the presence of *A. niger* IBP2013. Media volumes of coconut meal, *viz*., 5, 10, 15, 20, 25g were optimized for extracellular lipase production by *A. niger* IBP2013 at 72h of time interval. In present study, the fungal inoculation in 10mg media of volume of coconut meal gave the maximum extracellular lipase activity (27.4±0.56 U/mL min^{-1}) and specific activity (128 U/mg) while the minimum extracellular activity (10.8±0.36 U/mL min^{-1}) and specific activity (26.1 U/mg) were found with the use of 25g coconut meal as solid medium, but intermediate values were obtained with other concentration of coconut meal (Fig. 2). So, the 10g coconut meal as SSF was optimized with other optimized concentration by using *A. niger* IBP2013. The present findings are in accordance with the earlier findings of Adinarayana *et al*., (2004), who reported that adequate amount of substrate is necessary at specific time interval for optimal growth. The less lipase production in present study by *A. niger* IBP2103 in less solid medium might be due to less supply of nutrients for adequate production of fungus. In opposite at high substrate level, the less lipase production

might be due to less growth of fugal in comparison with substrate amount. Opposite to present study, in an earlier study 15g of substrate was found optimal for maximum fungal growth and lipase activity (Balagi & Ebenzer, 2008), whereas in present study, 10g of coconut meal was found best for further studies.

Various inoculum levels and biosynthesis of extracellular lipase: Various inoculum levels such as (0.5, 1, 1.5, 2.0, 2.5mL) were used with other optimized conditions, to find the best level for maximum lipase production. The same levels were also used by Vanot *et al*., (2002), under SST condition. Among different levels, 1mL level of inoculum gave the maximum lipase activity (28.7±0.96 U/mL min^{-1}) along with specific activity (124 U/mg), while the minimum lipase activity and specific activity (12.6±0.56 U/mL min^{-1} & 29.4 U/mg, respectively) was found when 2.5 ml inoculum was used (Fig. 2). For further studies 1ml inoculums along with other optimized conditions was used for further studies for maximum lipase activity using *A. niger* IBP2013 along with specific activity. Similar findings were found earlier by Gutarra *et al*., (2005), where 1mL inoculum was found best for maximum lipase activity along with specific activity in *Penicillium simplicissimum* through SSF. The less activity of lipase with lower level of inoculum might be due to inadequate production of fungus with specific time and less production of lipase at higher level of inoculum might be due to inadequate substrate for fungal growth (Iftikhar *et al*., 2015). However, they reported different results as where 2mL inoculum was found best for maximum lipase activity.

Various incubation temperatures and biosynthesis of extracellular lipase: *A. niger* IBP2013 was grown at different incubation temperature regimes for like 20, 30, 40, 50, 60ºC and production potential of the enzyme and specific activity were determined. The maximum lipase activity (28.5±0.30 U/mL min^{-1}) and specific activity (130.9 U/mg) were obtained at 40ºC incubation along with other optimized eco-cultural conditions, while the minimum lipase activity (14.4±0.56 U/mL min^{-1}) and specific activity (38.1 U/mg) was found at 60ºC. However, at other temperatures, intermediate values of lipase activity and specific activity were observed (Fig. 2). The temperature specific changes in lipase activity are due to the enzyme specificity to specific temperature (Lima *et al*., 2003). A decrease in enzyme activity at 60ºC might be due to denaturation or inactivity at high temperature (Lima *et al*., 2003). So, appropriate temperature is necessary for maximum activity of enzymes (Diaz *et al*., 2006) or inactivity. Similar findings were reported by Diaz *et al*., (2006) in fungus *Rhizopus homothallicus* for lipase production. However, some opposite reports available, where different temperatures were selected for maximum lipase activity but the type of fungus was different from present ones. For example, Ramani *et al*., (2010) reported maximum lipase activity with *Penicillium chrysogenum* at 30ºC, while Yuzbashev *et al*., (2012) reported 30-40ºC for maximum lipase production in *Rhizopus oryzae*. So, for further studies 40ºC was found best and utilized for further analysis.

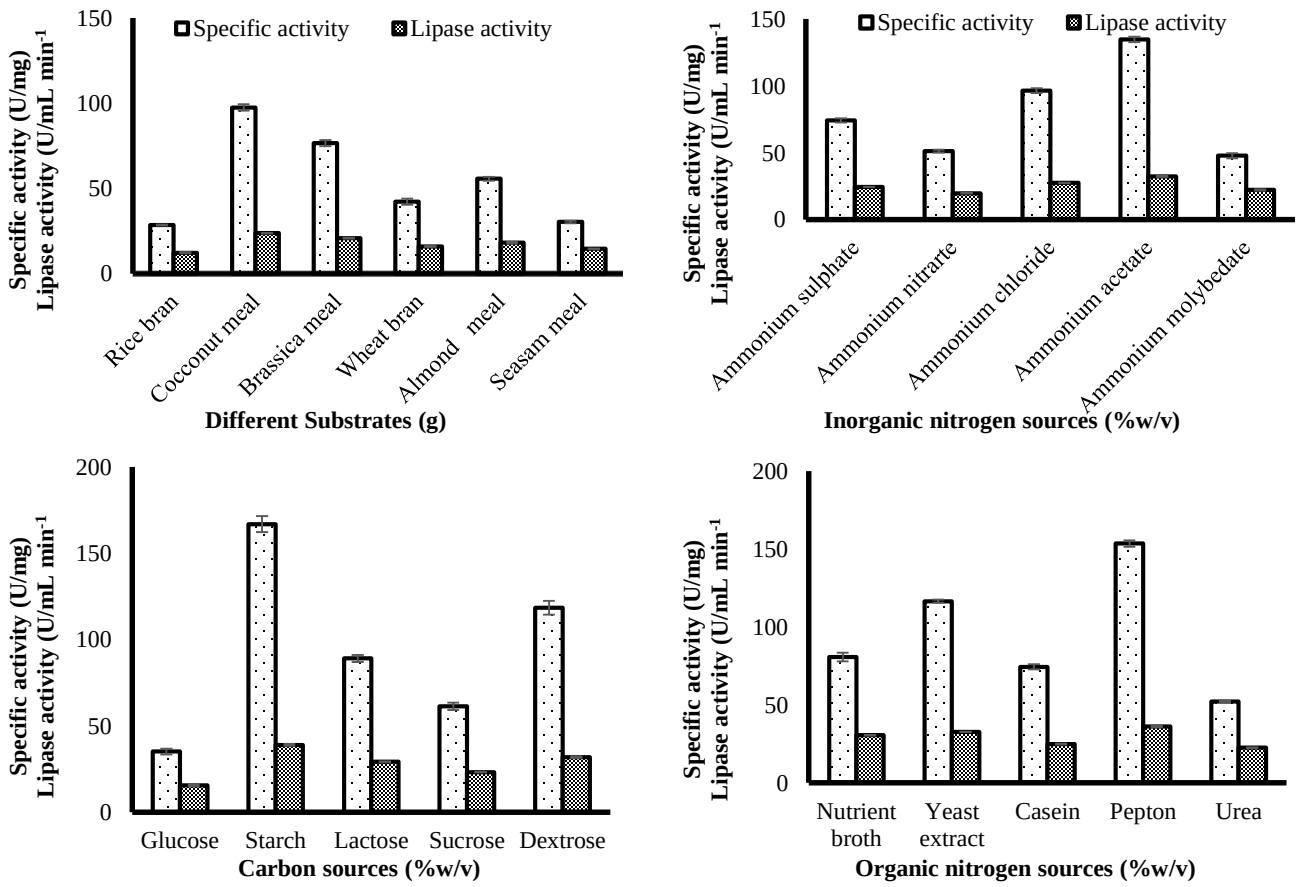

Fig. 1. Effect of growth media and media additives on extracellular lipase production by *A. niger* IBP2013 under solid substrate fermentation (Means ± S.E.).

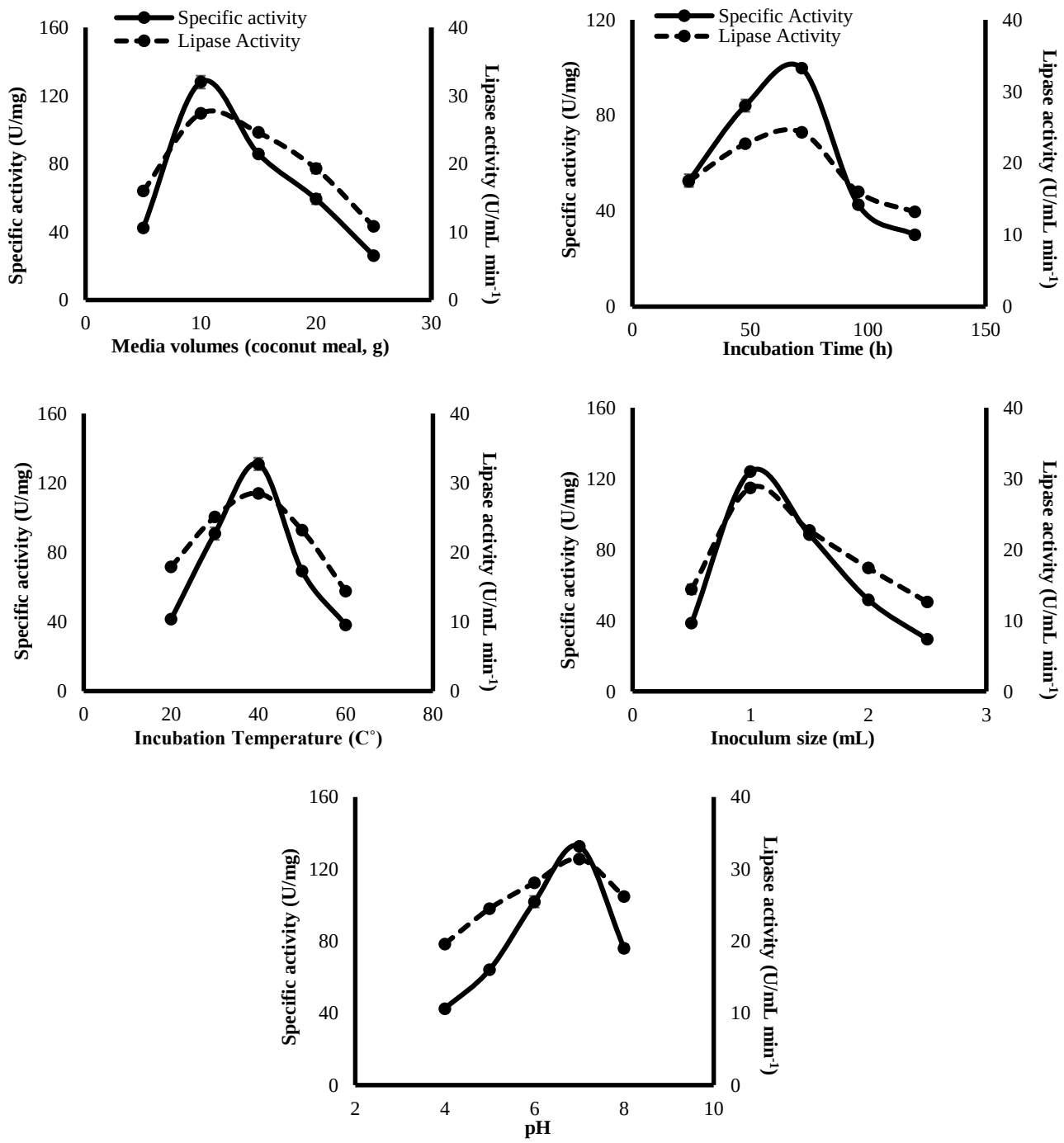

Fig. 2. Effect of various parameters (substrate weight; incubation time and temperature; inoculum size and pH) on extracellular lipase production by *A. niger* IBP2013 under solid substrate fermentation (Means ± S.E.).

Various pH levels and biosynthesis of extracellular lipase: Due to susceptibility of enzyme to pH, the medium pH is very crucial for better lipase biosynthesis. To obtain maximum lipase production at different pH with previously optimized conditions of growth media were utilized with *A. niger* IBP2013. Among different applied pH levels, the maximum lipase activity (31.4±0.25 U/mL min^{-1}) and specific activity (132.6 U/mg) was obtained at pH 7 revealing that fungus is neutrophilic. However, the minimum lipase activity and specific activity (19.6±0.25 U/mL min^{-1} and 42.5 U/mg, respectively) were observed at pH 4.0, while at other pH 5, 6 and 8 the intermediate results were obtained (Fig. 2). The findings of present study are parallel with the findings of Kamini *et al*., (2000) and Gupta *et al*., (2006), where the 4 and 7 pH was found best for maximum lipase production in *Cryptococcus* sp. and *Barkholderia multivorans* respectively. It is due to fact that enzymes show their stability at 4-7 pH and loss their activity at 40-60ºC at pH 8-10 (Salihu *et al*., 2012). However, Pandey *et al*., (2016) reported maximum lipase activity at 5-7 pH in cold and pH tolerant *Penicillium* spps. Furthermore, maximum lipase production was obtained at pH 5 (Gutarra *et al*., 2005) and pH 6.5 (Sun *et al*., 2008) in *Penicillium simplicissium* and *Rhizopus chinensisin* respectively. This difference might be due to the type of fungus and solid substrate used for culturing.

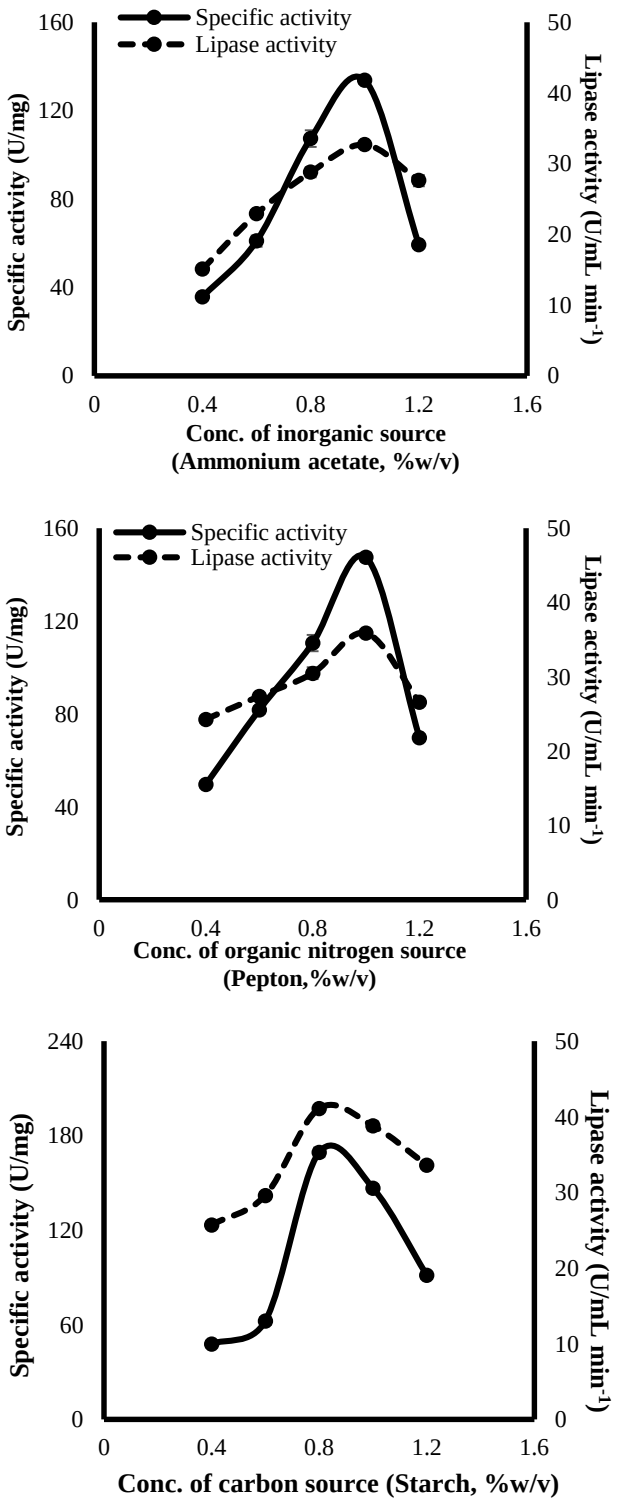

Fig. 3. Effects of various concentrations of media additives on extracellular lipase production by *A. niger* IBP2013 under solid substrate fermentation (Means ± S.E.).

Different inorganic N-additives and biosynthesis of extracellular lipase: The lipase production by fungus also depends on the available N for growth. In present study, among different N sources used for maximum fungus growth and lipase production, the maximum lipase activity (32.3±0.65 U/mL min^{-1}) along with specific activity (134.7 U/mg) was obtained by supplying with ammonium acetate at above best optimized conditions and minimum lipase activity (19.7±0.40 U/mL min^{-1}) and specific activity (51.2 U/mg) was obtained in the presence of minimum nitrate. However, other media showed intermediate results (Fig. 1). Similar results have been reported by Iftikhar *et al*., (2014), who found best lipase production when the culturing medium was supplied with 1% ammonium acetate. This might be due to behavior of lipase toward anion as per the investigations. In earlier study Burkert *et al*., (2004) reported ammonium nitrate as best additive for better lipase production in *Geotrichum* spp. In another study Lima *et al*., (2013) reported ammonium sulphate as best source of N for better lipase production.

Effect of various concentrations of optimized inorganic N-additive: For the maximum lipase biosynthesis, different levels of ammonium acetate as inorganic N source was used. The maximum lipase production/activity (32.7±0.2 U/ mL min^{-1}) and specific activity (133.7 U/mg) was obtained with 1% w/v concentration of ammonium acetate at previous optimized concentration. The minimum activity and specific activity (35.7 U/mg and 15.1±0.72 U/mL min^{-1}) was obtained with the medium supplied with 0.4% ammonium acetate but other levels of ammonium acetate gave intermediate values (Fig. 3). Similar findings were reported by Iftikhar *et al*., (2012), where also 1% level of ammonium acetate as inorganic N source was found better for lipase production.

Effect of various N-additives on the biosynthesis of extracellular lipase: Different organic N sources were also used for maximum lipase production and specific activity. The maximum lipase production and specific activity (36.2±0.89 U/mL min^{-1} and 153.5 U/mg) were obtained in the presence of urea as organic N source. Similar organic N source as that in present study were used by Hosseinpour *et al*., (2011), who found peptone as best nutrient additive medium for maximum lipase production. However, Gupta *et al*., (2007) and Iftikhar *et al*., (2012) found yeast extract and casein respectively as best and organic N additive for best lipase production.

Optimization of concentrations of organic N-additive: Results showed that among different levels of peptone applied for organic N sources, the 1% w/v was found best for maximum lipase production (35.9±0.70 U/mL min^{-1}) and specific activity ('147.5 U/mg) and minimum was found at 0.4% level of peptone (Fig. 3). In an earlier study, Iftikhar *et al*., (2014) found that 0.80% level of casein as best organic additive for maximum lipase production and specific activity in *Rhizopus oligoporus* Var. *Microporus* 11B63.

Effect of varying C-additives on the biosynthesis of extracellular lipase: Different sources of C-additives were applied to solid state fermentation using *A. niger* IBP2013 for better lipase production with previously optimized conditions. Results showed that maximum lipase units (39±0.89 U/mL min^{-1}) and specific activity (166.8 U/mg) was obtained with medium supplied with lactose while minimum lipase unit and specific activity (15.7±0.95 U/mL min^{-1} and 35.2 U/m) respectively was obtained in the presence of glucose (Fig. 1). The present results were found contrary to Gupta *et al*., (2007) and Kathiravan *et al*., (2012) where glucose found best carbon source for maximum lipase production in *Penicillium restrictum.*

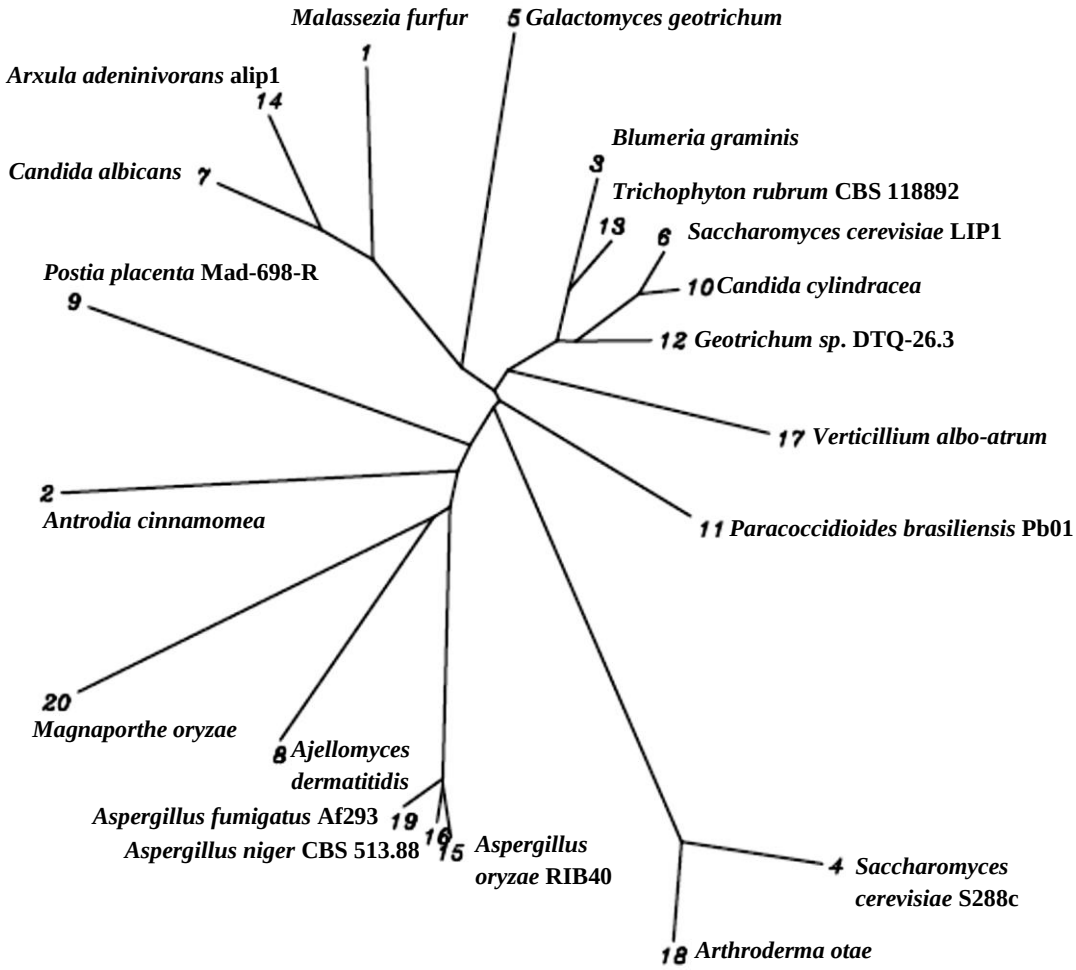

Fig. 4. Phylogentic relationship of LIP1 gene. 1= gi:73765554 2= gi:125503210 3= gi:190611653 4=gi: 296147419 5= gi: 410657 6=gi: 2852389 7= gi: 4096659 8= gi: 261199985 9= gi: 242221618 10= gi: 38565535 11= gi: 295665195 12= gi: 116563957 13= gi: 327305514 14= gi: 58651818 15= gi: 317139922 16= gi: 317026110 17= gi: 302423005 18= gi: 296813936 19= gi: 70990317 20= gi: 389638569.

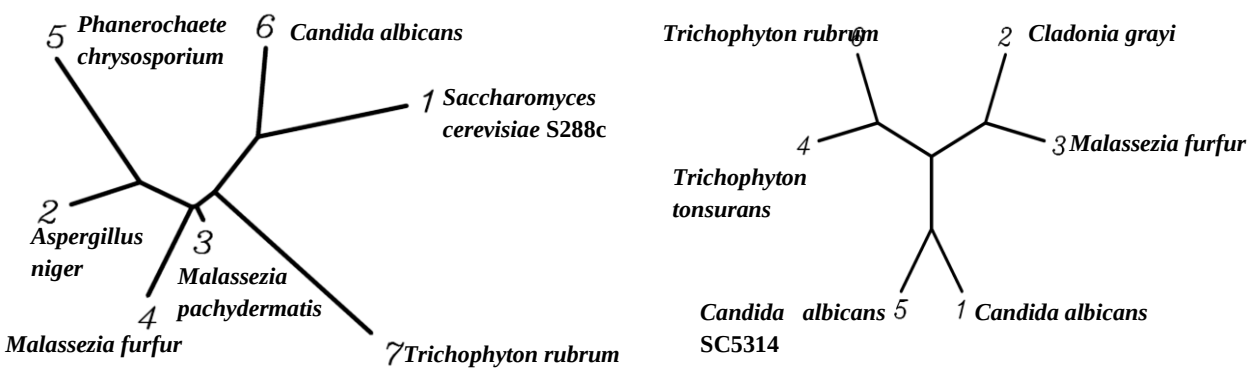

Fig. 5. (a) Phylogentic relationship of LIP2 gene (1= gi: 296146773 2= gi: 219963277 3= gi: 302393213 4= gi: 301963508 5= gi: 169277 6= gi: 6978035 7= gi: 110339270) (b) Phylogentic relationship of LIP3 gene 1= gi: 32141426 2= gi: 262479284 3= gi: 301963510 4= gi: 210076626 5= gi: 68465303 SC5314 6= gi: 110339272.

Effect of various regimes of C-additive on the production of extracellular lipase: Among different levels of lactose used for best lipase production under SSF using *A. niger* IBP2013 0.8% was found best for maximum lipase production and specific activity (41.1±0.40 U/mL min^{-1} and 169.5 U/mg respectively) under previously optimized conditions, while the minimum lipase production along with specific activity (25.7±0.95 U/mL min^{-1} and 47.8 U/mg) respectively was found 0.4% lactose supply (Fig. 3).

Phylogentic relationship of LIP1 gene: Gene sequence similarity method was employed to find out the interrelationship among various lipase producing strains.

Fungal strains were grouped based on the similarity and dissimilarity in their lipase gene sequences. In a group of four strains, the three fungal strains namely *Arxulaadeninivorans* alip (14= gi: 58651818), *Candida albicans* (7= gi: 4096659) and *Malassezia furfur* (1= gi: 73765554) were selected with similar gene sequence, while the strain *Galactomyces geotrichum* (5= gi: 410657) was selected as its own separate sequence. Similarly, another group of fual strains, namely *Candida cylindracea* (10= gi: 38565535), *Blumeria graminis* (3= gi: 190611653), *Geotrichum sp*. DTQ-26.3 (12= gi: 116563957), *Geotrichum* (6=gi: 2852389) and *Trichophyton rubrum* (13= gi: 327305514, CBS 118892) were selected similar gene sequence. Like that another fungal group comprised two strains with similar genomic sequence namely *Magnaporthe oryzae* (20= gi: 389638569) and *Ajellomyces dermatitidis* (8= gi: 261199985) were selected. Similarly, another group of similar strains like *Saccharomyces cerevisiae* S288c (4=gi: 296147419) and *Arthroderma otae* (18= gi: 296813936) was selected. Furthermore, based on similarity in evolutionary gene sequence strains *Aspergillus fumigatus* Af293 (19= gi: 70990317), *Aspergillus oryzae* RIB40 (15= gi: 317139922) and *A. niger* CBS 513.88 (16= gi: 317026110) were grouped together. However, fungal strains *Verticillium albo-atrum* (17= gi: 302423005), *Postia placenta* Mad-698-R (9= gi: 242221618), *Paracoccidioides brasiliensis* Pb01 (11= gi: 295665195) and *Antrodia cinnamomea* (2= gi: 125503210) showed no resemblance with the other lipase gene sequences, so grouped independently (Fig. 4).

Phylogentic relationship of LIP2 gene: Lipase 2 gene encoding lipase enzymes were searched in online gene sequence database and analyzed for their origin and evolutionary relationship. A group containing, *Saccharomyces cerevisiae* S288c (1= gi: 296146773) and *Candida albicans* (6= gi: 6978035) had similarity in genome sequences of *lip 2*. Similarly, fungal species *A. niger* (2= gi: 219963277) and *Phanero chaetechrysosporium* (5= gi: 169277) were also showed similarity in their *lip 2* gene sequences. Fungal strains as *Malassezia furfur* (4= gi: 301963508) and *Malassezia pachydermatis* (3= gi: 302393213) showed close resemblance in *Lip 2* sequence and probably have same evolutionary origin while *Trichophyton rubrum* (7= gi: 110339270) did not showed any resemblance with other sequences, thus could have independent origin (Fig. 5a).

Phylogentic relationship of LIP3 gene: Phylogenetic relationship based on *Lip 3* gene also studied through online tools of sequences analysis. The *Lip 3* gene sequences of various fungi were collected from gene sequence by data-mining and used to determine their evolutionary origin and relationship. Phylogenetic analysis based similarity between *Trichophyton rubrum* (6= gi: 110339272) and *Candida albicans* (1= gi: 32141426, SC5314) while *Lip 3* gene sequence of *Cladonia grayi* (2= gi: 262479284) had similarity with the gene of *Candida albicans* (5= gi: 68465303, SC5314). In the similar manner, *Malassezia furfur* (3= gi: 301963510) and, *Trichophyton tonsurans* (4= gi: 210076626) showed resemblance in their *Lip 3* gene sequence, so have probably same origin (Fig. 5b). The phylogeny of fungal strains was determined by comparing their genomic sequence of different lipase genes. It might be helpful to find out the evolutionary relationship between different lipase producing fungal species.

In conclusion, the fungal isolates with hyper lipase production ability in lipid rich conditions from various rotten food sources found having ability of thermo-tolerance with potential of biosynthesis of thermophillic enzymes which can be used for industrial employment. Overall, fungal strain *A. niger* IBP2013 found best for hyper lipase production under (SSF). Furthermore, this inherent potential of triglycerol acyl-hydrolases (lipases) production of the fungus was enhanced (73.41% la and 74.02% lsa) with eco-cultural optimization.

Conclusions and Recommendations

The findings of present studies show that the selected fungal strains of *A. niger* IBP2013 has potential to be used at industrial to be used at industrial and commercial levels to reduce the economic burden of Pakistan. The selected fungal strain *A. niger* IBP2013 with potential of thermos-stability with increased enzymes production could be a good candidate for lipase production on industrial scale. Although there is still required to explore the genetic potential of this native fungal strain by amplifications of lipases genes, identification of number of copies of the gene in genome etc. and monitoring the modulation of these genes in accordance with our optimized growth culture conditions under SSF.

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