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MORPHO-MOLECULAR IDENTIFICATION OF A NOVEL *ASPERGILLUS* SPP. AND ITS CULTURAL OPTIMIZATION FOR LIPASES PRODUCTION

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Abstract

Different lipid rich products were used to obtain oil degrading fungal isolates. The isolates were codified for referral to our culture bank and compared for their lipolytic potential. Amongst the isolates, MBL-1412 isolated from the cooked "sliced *cicer arietinum*" (Channa Daal) was found to be a potent hyper-producer and was optimized for lipase production under solid state fermentation. Initial systematic treatment based upon micrometric data and consultation with the standard monographs and fungus ended up with its identification as *Aspergillus* sp. The identification confirmed that the fungus belongs to genus *Aspergillus*, by DNA barcoding marker like 18S rRNA gene sequence.Later, the sequence was registered with accession no. KM924434 in the public nucleotide library (genbank) of NCBI. Fungal culture was maintained on 2% potato dextrose agar (PDA) during the study. Diverse substrates of agricultural byproducts under varied incubation temperature, time interval, inoculum level and different pH of diluent were used as parameters of optimization for hyper-production of lipases. Different carbon and nitrogen sources as additives of culture medium were applied for enhancement of lipase production. Almond meal (10g) with inoculum level @1.5 mL after 48 h of time course at 50ºC and 6 pH were selected to be the best eco-cultural conditions for optimal lipases production by *Aspergillus sp.* MBL-1412. Supplementary additives of nitrogen and carbon sources to the basal substrate improved lipases production appreciably. Ammonium chloride (1%) as inorganic nitrogen source, nutrient broth (0.8%) as organic nitrogen source and starch (0.8%) as carbon source were found as best media additives for enhanced extracellular lipases yield.

Key words: Fungal Lipase production, 18S rRNA, *Aspergillus* Sp., DNA barcoding marker, Solid state fermentation.

Introduction

In last two decades, the identification of fungi through DNA sequences (DNA barcoding) has been adopted as a pre-requisite and essential part of the researches in the field of fungal ecology and diversity. The data generated in this regard establishes new horizons of research in these fields. As a DNA barcoding marker, the internal transcribed spacer (ITS) of genomic DNA (gDNA) is considered as most suitable marker for fungal identification till single taxon level. For this purpose, more than 10,000 fungal ITS gDNA sequences are already deposited in International Public Nucleotide Sequence databases (Nilsson *et al*., 2009). These sequence databases providing a huge reference material for fungal taxa identifications. Identified fungi provides sound basis of their inherent potential for the production of industrially important enzymes.

Enzymes have now become the core part of researcher's choice of work for their multiple industrial applications. This catalytic machinery accelerates the chemical and biological reactions. Moreover, these tiny molecules are being employed in different fields like agriculture and industry in Pakistan, notably food processing, leather, detergent and chemical industries etc. (Houde *et al.*, 2004; Couto and Sanoroman, 2006; Liu *et al*., 2012; Purich, 2010). Among biocatalysts, lipases are the third major enzyme group on account of its employment for industrial usage (Saxena *et al.*, 2003).

Lipases, triglycerol acyl-hydrolases (E.C. 3.1.1.3), split the triacylglycerol into glycerol and fatty acids. Ester formation in liquid media is another role of lipases. The lipases are inherent part of animal, plant and microbial metabolism. However, microorganism like bacteria and fungi are preferred for low cost and efficient commercial lipase production. Fungi are further advantageous over bacterial sources on account of their ready availability and stability as well as ease of culturing (Houde *et al.*, 2004; Gombert *et al.*, 1999; Salihu *et al.*, 2012). *Aspergillus* sp., are filamentous fungi and have great commercial value. They can be attained from varied environmental surroundings such as rottening food or organic matter rich soil materials etc. *Aspergillus* sp., have been reported for commercial production of extracellular lipase (Maccabe *et al.*, 2002). Beside other fungi like *Penicilium* sp, *Fusarium* sp., *Rhizopus* sp., *Alternaria alternata*, etc. our research group has optimized eco-cultural optimization for *Aspergillus nidulans and Aspergillus niger* for commercial lipase production (Maia *et al.*, 2001; Lima *et al.*, 2003; Iftikhar *et al*., 2010).

Screening of fungi for targeted enzyme production is being done by using different techniques (Vakhlu *et al.*, 2006), such as solid substrate fermentation (SSF) and submerged fermentation technique (SmF) (Soccol & Vandenbergh, 2003; Diaz *et al.*, 2006). Solid state fermentation is preferable over submerged fermentation due to the involvement of low-cost mechanization, less energy inputs and larger titers of required enzymes output (Aguilar *et al.*, 2001; Soccol & Vandenbergh, 2003). Production potential of fungi can further be enhanced by optimizing eco-cultural conditions like finding appropriate substrate, amount of substrates, incubation period, inoculum level, temperature and pH (Gutara *et al*., 2009) as well as addition of some supplemental organic and inorganic nitrogen and carbon material to culture media (Fickers *et al.*, 2004; Lima *et al.*, 2003).

Conventional mechanical industrial set up being replaced with innovative transformations and bio-processing is the most favored option. Lipases have vast application potential in detergent, food and pharmaceutical industry. Despite the native conducive eco-climatic conditions for inhabitation of thermo-tolerant fungi having thermostable genetic potential, all the industrial need of enzymes is being met through import. This is an additional burden on dwindling economy of Pakistan. This is the high time to explore the indigenous fungi for local enzyme production. The work has been designed to identify indigenous fungal flora through morphological examination and DNA barcoding markers. The identified fungus was further exploited for commercial and industrial scale lipases production. The research will be helpful to go for more comprehensive studies of indigenous fungi with inherent potential for the production of enzymes of industrial significance.

Materials and Methods

Isolation of fungi: Fungal strains were isolated from different decaying food material (Fig. 1) by incubating the rotten food items at room temperature. After one week, visible colonies were shifted to Potato Dextrose Agar (PDA) slants (Iftikhar *et al*., 2011). Primarily, the isolated cultures were codified with Alpha-numeric of MBL (Mycology Biotechnology Laboratory #). Malt extract agar plates (malt extract 20g/L; agar-agar 20g/L) were used to isolate the fungi from rotten food items. Hyper-producers were then identified by using microscope (LABOMED, Lx 400) and lipase production potential of each isolate was determined after following Iftikhar *et al*. (2010). The fungal cultures were maintained on 4% PDA slants after following Iftikhar *et al*., (2010).

Systematic treatments for identification of fungal isolate: The prepared slides were examined by using microscope (LABOMED) fitted out with camera (LABOMED, iVu 1500). Morphological data of mycelium, colonies and reproductive structure characteristics of fungal samples was obtained by using different lens (10X, 40X etc). Images were taken with the attached camera. Identification was made by using standard literature and monographs. Fungal attributes like shape of the colony, shape and color of conidiophores, color of fungus, shape of sporangia, color and shape of conidia/spores as well as hyphal morphology, etc., were taken as parameters of identification.

DNA barcoding studies: The identified fungal hyper lipase producer was utilized for extraction of gDNA as described by van Kan *et al*., (1991). Briefly, one gram (fresh weight) of the fungus was ground to fine powder in liquid nitrogen (N_2). The extraction of gDNA was performed with preheated at 65°C, 3 ml extraction buffer (0.5 M NaCl, 1% SDS, 10 mM Na_2EDTA and 10 mM Tris-HCl, pH 7.5). After 10 min of incubation at room temperature, the suspension was centrifuged (15000 g) with bench top centrifuge for 10 min to remove the debris, and the aqueous gDNA was separated by phase separation with phenol (pH 8) and extracted with 2 ml CI (chloroform and isoamyle alcohol, 24:1, v/v). The supernatant was treated with RNase A (10 µl, 10 mg ml^{-1}) at 37°C for 15 min. RNase A was extracted with phenol and CI. gDNA was precipitated with isopropanol (100%) and washed with 70% ethanol. The extracted gDNA was sent to Macrogen, Korea (http: //www. macrogen.com/) for sequencing with 18S universal primers (ITS1 and ITS4, White et al., 1990). The sequences were aligned and removed any ambiguity, if present. The sequence was stored in the public nucleotide database of NCBI (https://www.ncbi.nlm.nih.gov/genbank/).

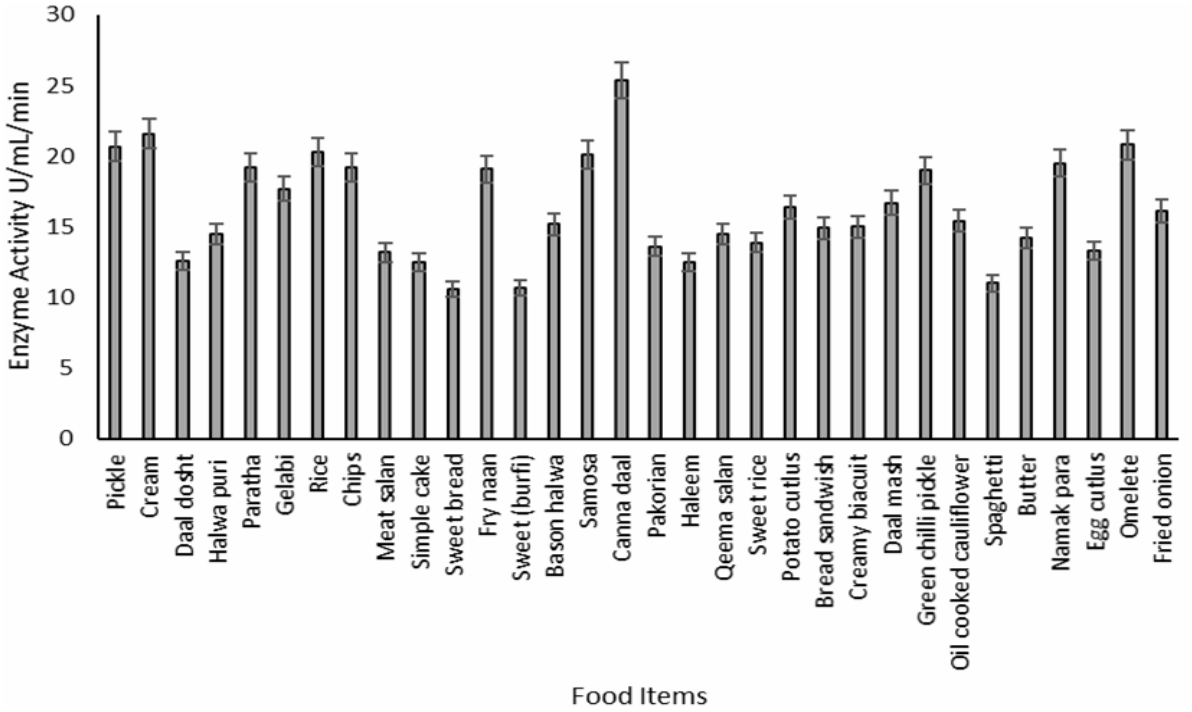

Fig. 1. Comparison of different food items for extracellular lipase production through solid state fermentation technique.

Fermentation technique: The fungal strains were subjected to solid state fermentation for lipases production. After adding solid substrate of 10g (brassica meal) and 10 mL of distilled water to each cotton-wool plugged conical flask was autoclaved at 121°C having 15 lb/inch^2 pressure for 15 minutes. After cooling, each flask was loaded with 1mL of inoculum under aseptic conditions. The flasks were then incubated at 30 C for 48 hours. The fermenting broth in the flasks was used to estimate the lipase production in triplicate as described by Iftikhar *et al*., (2010**).**

Enzyme assay: After 48 hours of incubation period the flask's material were centrifuged for 15 minute at the speed of 5000 rpm and supernatant were assayed for extracellular lipase activity as units of lipase produced after following Kempka *et al*., (2008). Lipase unit is the amount of enzyme which releases one micromole fatty acid per minute under specified assay conditions. Specific activity was calculated on the basis of the enzyme produced per unit of total proteins released.

Statistical analysis: All the experiments were statistically analyzed by using a computer software Co-Stat CoHort Software version 6.4. Duncan multiple range (DMR) applied under one way ANOVA

Results and Discussion

Screening of hyper-producer: Various native fungal strains were isolated from 32 rotten food stuffs such as basin halwa, sweets (burfi), pickle, sweet bread, omelet, rice cooked in oil, roasted minced meat, cooked spaghetti, cooked "sliced *Cicer arietinum*" (channa daal), creamy biscuit, Lentil (daal) fried in oil, meat cooked in oil, fried onion, butter, oil cooked cauliflower, cake, butter, samoosa, and fried naan etc (Fig. 1). These fungal colonies were grown on PDA and kept at 30˚C. Visible colonies were seen after one week.

In the present research, various strains were isolated from diverse food stuffs and tagged with the name of the food stuff (Figure 1). *Aspergillus* sp. (MBL-1412) isolated from channa daal, found to be the hyper producer of lipase units (25.4 U/mL/min) and minimum units (10.6 U/mL min^{-1}) were obtained from sweet fried bread (Fig. 1). Based upon the results, *Aspergillus* sp. (MBL-1412) was subjected to optimization of eco-cultural conditions for lipase yield under solid state fermentation. Different environmental parameters like various substrates, conc. of substrates, diverse time intervals, inoculum size, pH and temperature were found to have an effect on lipase biosynthesis.

Brief description of the *Aspergillus* sp. (MBL-1412): The fungal appearance after growing on PDA was plain green, cream-buff or honey-yellow. Conidial heads short, columnar and biseriate. Conidiophore was short and contain smooth wall with brownish color. Vesicles hemispherical and metulae were present on vesicles. Conidia globose, sub hyaline and greenish in mass with rough-walled. Cleistothecium surrounded by yellowish and scattered hyphae, ascospores reddish-brown and have smooth walls (Plate 1).

Identification of selected fungal strain: Using morphological and reproductive attributes, hyper lipase producing fungi as mentioned above was identified as genus *Aspergillus*, codified for our data and culture bank as MBL-1412.

Plate 1. *Aspergillus* sp. (MBL-1412): Petriplate showing fungal appearance on potato dextrose agarmedium

Molecular studies of hyper-producer: To identify the fungi on the genetic basis, 18S rRNA gene sequence is being widely utilized as a molecular marker. The whole mass of mycelium from the colony was used to extract gDNA. 18S rRNA gene was targeted for sequence based identification. The two universal primers ITS1 and ITS4 were used as represented in the Fig. 2. The obtained sequence was stored in the genbank with the accession no. KM924434. The sequence analysis suggested its similarities with *Aspergillus* sp.

Eco-cultural conditions for lipase productions: To evaluate the lipases production and eco-cultural optimization, the effect of diverse types of substrates was taken as the 1st criterion and *Aspergillus* sp. (MBL-1412) was incubated with brassica meal, rice bran, wheat bran, almond meal, and coconut meal under solid state fermentation conditions. Almond meal gave the highest lipase yield and the specific activity, 25.9±0.37^{a} U/mL/min., 134.7 U/mg, respectively (Fig. 3). Wheat bran showed the lowest enzyme activity (11.2±0.65^{e} U/mL/min) and specific activity of (23.8U /mg). Almond meal had already been selected as the best agro-based substrate for lipase production for other fungi by Iftikhar *et al.,* (2010). The reason might be that almond meal fulfilled the best nutrient requirements for fungal growth.

Optimization for incubation period: Data pertaining to incubation of *Aspergillus* sp. (MBL-1412) *i.e*., 24, 48, 72, 96 and 120 hours through solid state fermentation is presented as Fig. 4. Enzyme production and specific activity were maximum (26.2±0.45 U/mL/min, 135.8U/mg, respectively after 48 hours (Fig. 4). While both these parameters were dropped down to the level of 10.8±0.51 U/mL/min and 22.8U/mg respectively, after 120 hours. Similar results had already been reported by Faloni *et al*., (2006) and Kempka *et al*., (2008).

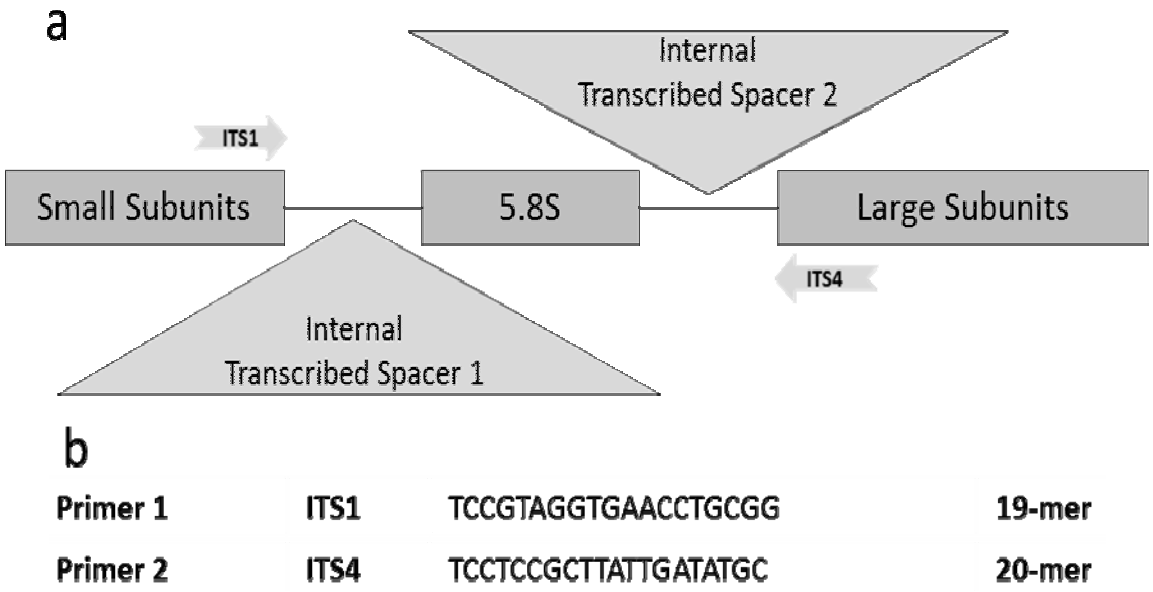

Fig. 2. A general map of 18S rRNA gene of fungi with specific positions of 18S universal primers ITS1 and ITS4 (a) with their respective sequences (b).

Effect of substrate concentration: Various substrate concentrations (5, 10, 15, 20, 25 g) were evaluated under solid state fermentation under previously optimized conditions for *Aspergillus* sp. (MBL-1412). A comparative look at lipase production and specific activity showed that 10 g of almond meal gave the maximum lipase yield (30.4±0.62^{a} U/mL/min) and specific activity of 28.8U/mg at incubation period of 48 hours (Fig. 5). Minimum value of lipase activity (11.3±0.15^{e} U/mL/min) and specific activity (26.4U/mg) respectively were obtained with 25 g of fermenting medium (Fig. 5). The results are similar to Gutarra *et al*., (2005). The probable reasons of this drop of efficiency of the fungus at higher substrate concentration might be that high amount of substrate with low inoculum level might have disturbed the nutrient to inoculum ratio. For this reason, the inoculum size was selected as the next parameter of the study.

Effect of spore inoculum size: To evaluate the effect of size of inoculum, 0.5, 1, 1.5, 2, 2.5 mL of inocula were applied and the resultant comparison of enzyme production and specific activity is given as Fig. 6. Maximum lipase activity of (30.1±0.75^{a} U/mL/min) and specific activity (155.5 U/mg) were observed at 1.5 mL inoculum. With the increase of inoculum size, the enzyme activity as lipase activity (10.9±1.62^{e} U/mL/min) and specific activity (25.3 U/mg) dropped and probable reason might be that at high inoculum level substrate concentration become low as compared to available fungal propagules. These findings confirm our hypothesis for previous parameter. However, these results are slightly different from earlier reports where a linear increase in enzyme production and specific activities had been reported with increase in inoculum size (Gutarra *et al.,* 2005).

Effect of incubation temperature: Temperature of fermenting substrate plays a crucial role in enzyme catalysis. Various incubation temperatures ranges from 20-60°C were applied to *Aspergillus* sp. (MBL-1412) growing under previously optimized eco-cultural conditions. The data was used to conclude their effect on lipases production and specific activity under solid state fermentation. Maximum lipase activity was observed at 50°C after 48 hours of incubation period (39.0±0.67 U/mL/min) and specific activity was (143.3 U/mg) while lipases production was minimum 13.6±0.20 U/mL/min and 32.4 U/mg after 20 hours, respectively (Fig. 7). The enzyme production decreased with the increase of incubation temperature. These results are in line with Falony *et al*., (2006). Our findings also confirm the results of Maia *et al*., (2001); Lima *et al*., (2003).These workers are of the opinion that drop in fungal efficiency in enzyme production might be due to the reason that low temperature limits the fungal growth by direct involvement of environmental temperature in metabolic biosynthesis.

Effect of pH of diluent: A pH range of 4-8 was given for optimization of solid substrate fermentation mechanism of *Aspergillus* sp. (MBL-1412). Maximum lipase activity 38.5±0.36^{a} U/mL/min and specific activity of 156.3 U/mg were achieved at pH of 6 on previously optimized conditions (Fig. 8). The results are in line with Anbu *et al*., (2011). Minimum lipase activity (19.5±0.40^{e} U/mL/min) and specific activity (33.6 U/mg) were observed at pH 4. A pH range of 5-6 had already been optimized for maximum lipase activity by using fungal strain as *Penicillium simplicissimum* (Gutarra *et al*., 2009). However, Sun & Xu, (2008) reported maximum activity at 6.5 pH for *Rhizopus chinensis*.

Optimization for diluent additives: Various additives to the diluent such as organic & inorganic nitrogen and carbon sources were optimized to boost up the lipase synthesis.

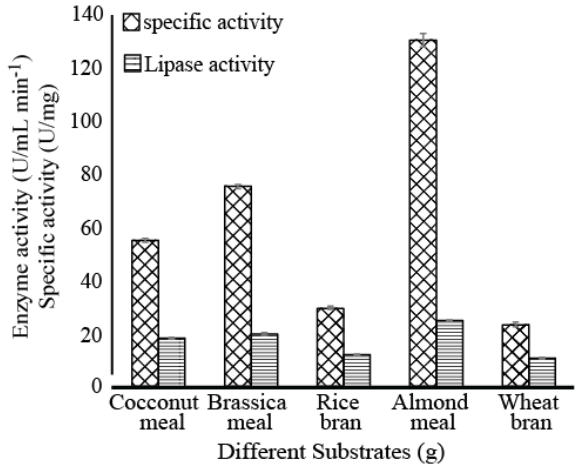

Fig. 3. Comparison of different substrates for extracellular lipase production by *Aspergillus* sp. (MBL-1412) under solid state fermentation.

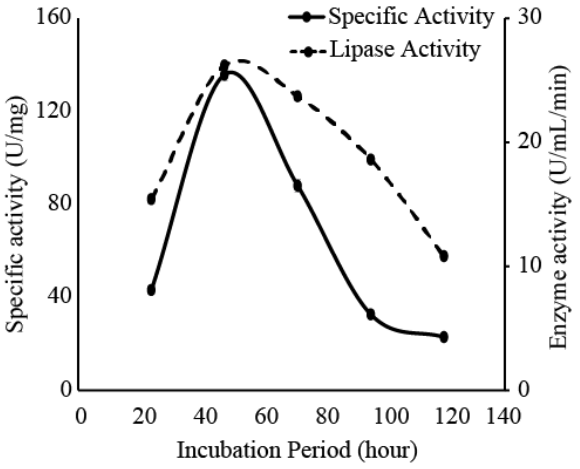

Fig. 4. Comparison of different incubation period on extracellular lipase production by *Aspergillus* sp. (MBL-1412) under solid state fermentation.

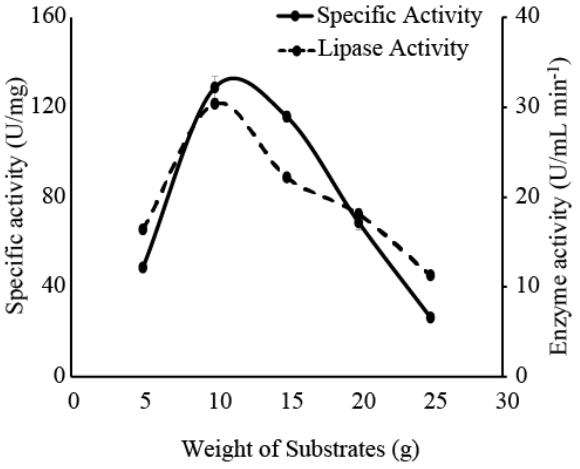

Fig. 5. Comparison of different quantity of substrate on extracellular lipase production of *Aspergillus* sp. (MBL-1412) under solid state fermentation.

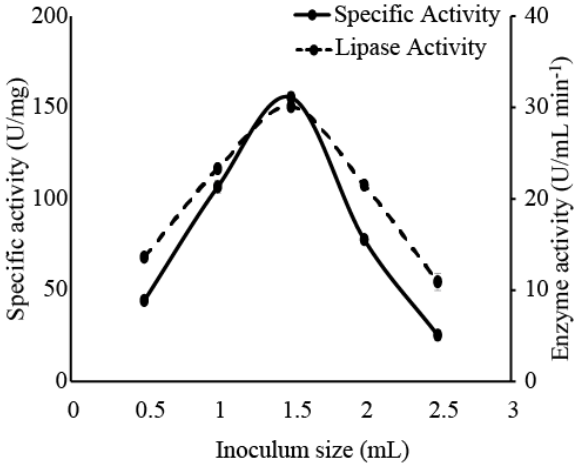

Fig. 6. Comparison of different inoculum size on extracellular lipase production of *Aspergillus* sp. (MBL-1412) under solid state fermentation.

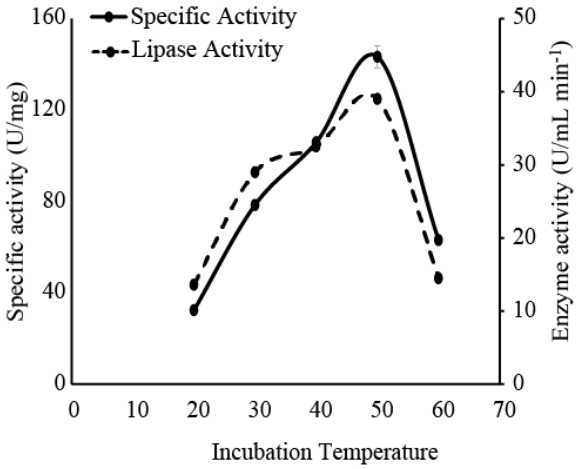

Fig. 7. Comparison of different incubation temperatures on extracellular lipase production of *Aspergillus* sp. (MBL-1412) under solid state fermentation.

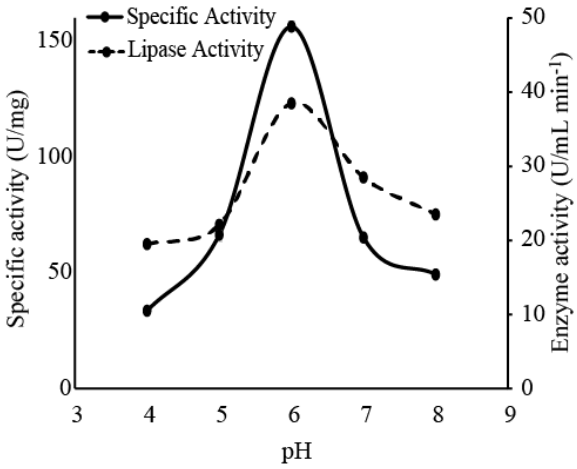

Fig. 8. Comparison of different pH on extracellular lipase production of *Aspergillus* sp. (MBL-1412) under solid state fermentation.

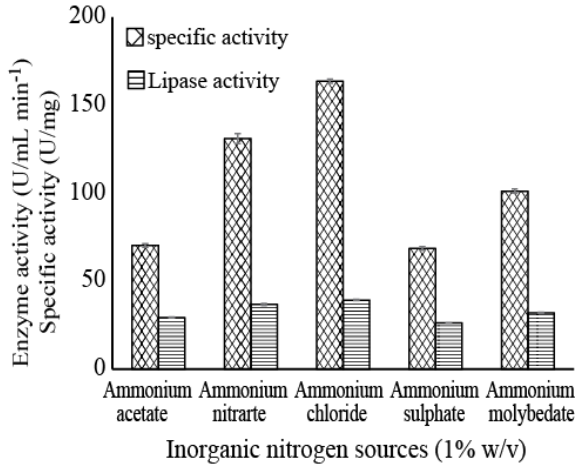

Fig. 9. Effect of inorganic nitrogen sources on extracellular lipase production by *Aspergillus* sp. (MBL-1412) under solid state fermentation.

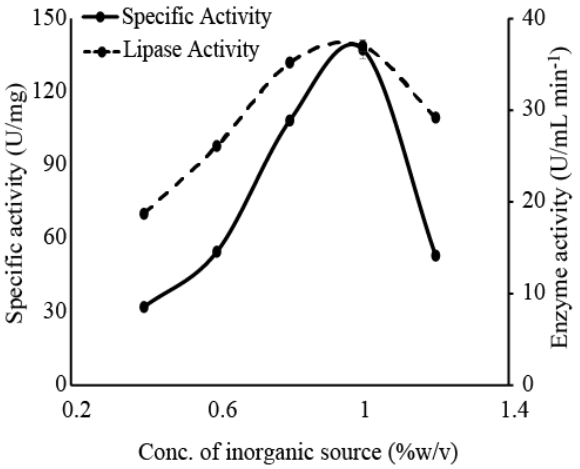

Fig. 10. Effect of concentration of inorganic nitrogen source on extracellular lipase production by *Aspergillus* sp. (MBL-1412) under solid state fermentation.

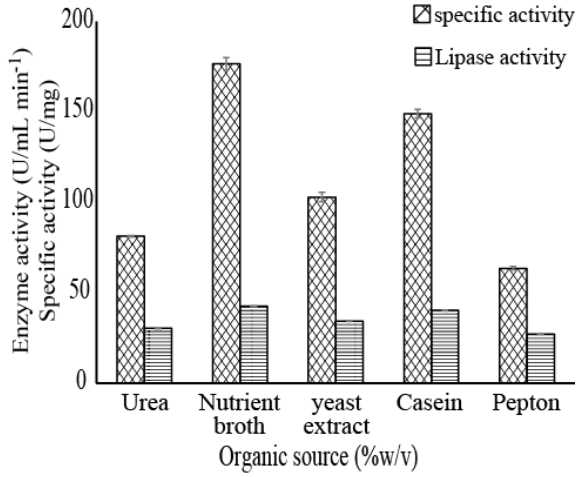

Fig. 11. Effect of organic nitrogen source on extracellular lipase production by *Aspergillus* sp. (MBL-1412) under solid state fermentation.

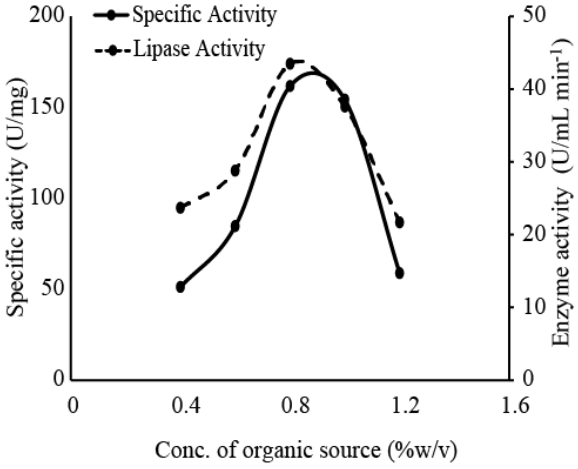

Fig. 12. Effect of concentration of organic nitrogen source on extracellular lipase production by *Aspergillus* sp. (MBL-1412) under solid state fermentation.

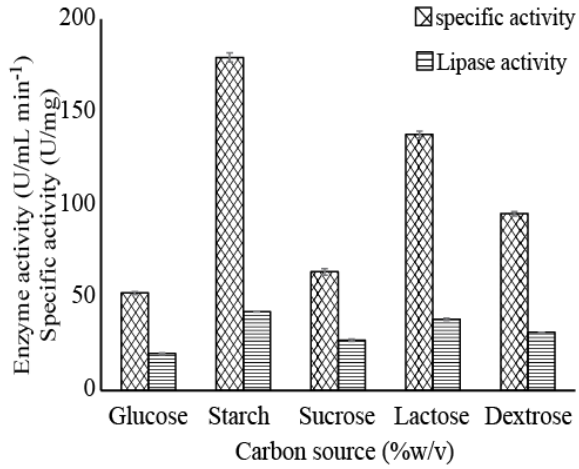

Fig. 13. Effect of carbon source on extracellular lipase production by *Aspergillus* sp. (MBL-1412) under solid state fermentation.

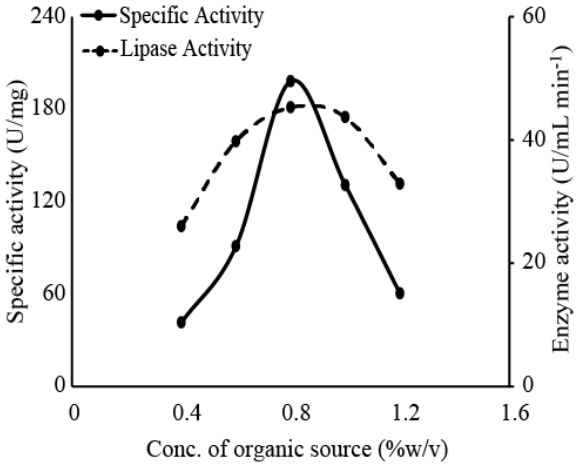

Fig. 14. Effect of concentration of organic carbon source on extracellular lipase production by *Aspergillus* sp. (MBL-1412) under solid state fermentation.

Inorganic nitrogen sources: To find the highest lipase production various nitrogen additives @1%, including NH_4NO_4, $(NH_4)_2SO_4$, NH_4Cl, $(NH_4)_6MoO_{24}$ and NH_4CH_3COO, were evaluated. Ammonium chloride (NH_4Cl) yielded the best enzyme units and specific activity which was 39.3±0.66[a] U/mL/min and 163.6 U/mg, respectively (Fig. 9). Ammonium sulfate gave the minimum activity both interms of enzyme activity (26.3±0.15[e] U/mL/min) and specific activity (68.6U/mg) (Fig. 9). Iftikhar *et al.,* 2010 reported ammonium salts as the best inorganic nitrogen sources for optimal lipases production under solid state fermentation conditions for *Rhizopus oligosporous.*

Optimization studies for concentration of inorganic nitrogen: Different concentrations of ammonium chloride (NH_4Cl) *i.e.*, 0.4, 0.6, 0.8, 1, 1.2% of the fermentation medium were applied. Ammonium chloride applied @ 1% further improved the enzyme yield (37±1.12[a] U/mL/min) and specific activity of (137.4 U/mg) (Fig. 10). Low concentrations of ammonium chloride (0.4%) with enzyme activity (18.7±0.64[e] U/mL/min) and specific activity of (32 U/mg) (Fig. 10).

Organic nitrogen sources: For optimization of this parameter urea, peptone nutrient broth, casein and yeast extract were subjected as additive to basal substrate under previously optimized eco-cultural conditions (Fig. 11). Nutrient broth yielded maximum lipase units (42.6±0.31[a] U/mL/min) and specific activity (176.7 U/mg) while peptone showed minimum lipase yield (27.1±0.35[e] U/mL/min) and specific activity (63.6 U/mg). These are unique findings and other researchers reported urea (Kathiravan *et al.,* 2012) and yeast extract (Gupta *et al*., 2007) to be the good organic nitrogen additive for optimal lipase production.

Optimization studies for concentration of organic nitrogen: Different concentrations of optimized organic nitrogen additive *i.e.*, 0.4, 0.6, 0.8, 1, 1.2% of the fermentation medium were applied. Nutrient broth applied @ 0.8% showed the maximum productivity with enzyme activity of 43.5±0.25[a] U/mL/min and specific activity of 161.7 U/mg (Fig. 12). Minimum activity of 21.7±0.81[e] U/mL/min and specific activity of 58.9 U/mg was recorded when nutrient broth was added to fermenting medium @1.2% (Fig. 12). Likewise, other results in the literature characterized that 0.8% casein by (Iftikhar *et al*., 2010) offered better lipase biosynthesis as organic nitrogen source from *Rhizopus oligosporous*.

Optimization studies for additional carbon source: Carbon sources like dextrose, sucrose, lactose, starch, and glucose @ 1% were subjected to the fermentation substrate to determine the best lipases yield. Starch as carbon source gave maximum lipases production. Enzyme units of 42.4±0.10 U/mL/min and specific activity of 179.4 U/mg was noted in the presence of starch while enzyme activity of 19.9±0.72[e] U/mL/min and specific activity of 52.5 U/mg was examined in the presence of glucose (Fig. 13). The rest of the additives yielded between these two extremes. Therefore, starch was selected as best mediator. Other researchers reported diverse carbon sources as best media additive like lactose and glucose (Gupta *et al.,* 2007).

Concentration optimization of carbon source: Starch @ 0.4, 0.6, 0.8, 1, 1.2% were added to the basal substrate. Maximum production of enzyme units (45.3±0.58[a] U/mL/min) and highest specific activity (198.2 U/mg) was recorded when starch was used as additive @ 0.8% (Fig. 14). Minimum lipase production (26±0.59[e] U/mL/min) and specific activity (41.5 U/mg) was observed at 0.4% starch (Fig. 14). There are varied reports for differential different concentrations of carbon sources as best production booster for lipases using different fungi (Iftikhar *et al*., 2010; Gupta *et al.,* 2007).

Conclusion

From the above results, it can be concluded that *Aspergillus* sp. (MBL-1412) sequence registered in NCBI database can be used as a reference for identification of fungi. Further, it could be a candidate for lipases yield, if kinetic parameters of its lipases qualify for industrial employment in terms of thermostability and thermophilicity.

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