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ISOLATION, IDENTIFICATION OF AN AXENIC FUNGAL ISOLATE OF *ASPERGILLUS* SP. (MBL-1511) AND ITS SUBSEQUENT IMPROVEMENT FOR ENHANCED EXTRACELLULAR LIPOLYTIC POTENTIAL THROUGH MONOCULTURE FERMENTATION

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Abstract

The present investigation was conducted for extracellular lipases production. One hundred and forty samples of fungi were isolated from different environment and food samples. Among all the isolated cultures, an isolate obtained from chicken roasted in oil (MBL-1511) gave the highest extracellular lipase through SSF. Hyper producer strain (MBL-1511) was morphologically identified. A morphologically identified isolate of *Aspergillus niger* (MBL 1511) was verified by DNA barcoding marker like 18S rRNA gene sequence. The sequence of *Aspergillus niger* (MBL 1511) was registered with accession no. [GenBank: KP172477] in the public nucleotide library (genbank) of NCBI. The selected hyper producer of *Aspergillus niger* (MBL-1511) strain was subjected to physical and chemical mutagenic treatments to improve its lipolytic potential. Proximate analysis confirmed brassica meal as the best basal substrate with the lipases potential of 10.67±0.01 IU/mL (wild) and 19.58±0.04 IU/mL (mutant). The optimum conditions for the maximized extracellular lipases production were 1.0 mL inoculum at 30°C after 72 h at pH of 6.2. Finally, a potent mutant of *A. niger* [MBL-1511^{SA-4}(150 min)] with an increased activity of 161 % over the wild strain was obtained when olive oil was used at 1% (v/v) concentration.

Key words: Fermentation, *Aspergillus*, Lipases, Bioprocessing, Barcoding.

Introduction

Lipases, triglycerol acyl-hydrolases (E.C. 3.1.1.3), split the triacylglycerol into glycerol and fatty acids. Fungi are preferred organisms for industrial scale production of enzymes because these are easily cultured, occupy little space, and multiply rapidly. *Aspergillus niger* is an important fungi used for production of lipase enzyme. Lipase is the third major enzyme group due to its production potential for industrial usage especially in detergent and pharmaceutical industry (Liu *et al*., 2012).

Fungal strain improvement and medium optimization is essential for the overproduction of the lipase, as the quantities produced by wild strains are usually too low. The spectacular success examples of strain improvement in industry are mostly attributed to the extensive application of mutation and selection of microorganisms (Bapiraju *et al*., 2004). For last two decades, identification of industrially important strains by DNA bar coding has received the attention of microbiologists. As a DNA barcoding marker, the internal transcribed spacer (ITS) of genomic DNA (gDNA) is the most suitable marker for fungal identification till single taxon level. Genetically identified fungi provides sound basis of their inherent potential for the production of industrially important enzymes (Nilsson *et al*., 2009).

In Pakistan, to meet the industrial demand, lipase enzymes are imported from other countries. So, it is necessary to produce lipases by using cheap fungal sources. The objective of the present work is to identify indigenous fungal flora by DNA barcoding markers and strain improvement through physical and chemical mutagenesis. Moreover, the wild strain of *Aspergillus niger* (MBL-1511) was exploited for improving its lipolytic potential by solid state fermentation (SSF) technique. The results of the proximate analysis of the basal substrate was also aimed which would be helpful in designing the medium for commercial production of lipases.

Materials and Methods

Isolation and screening of lipolytic fungi: One hundred and forty (140) samples of fungi were collected from different environment and food samples such as air, bread, butter, chicken roasted in oil, decaying fruits, decaying vegetables, fish roasted in oil, fry potato, pickle, pizza and soil by serial dilution method (Akano & Atanda, 1990). The fungal isolates were screened quantitatively through solid state fermentation (SSF) (Gopinath *et al*., 2005). Fungal hyper producer strain was selected and assigned the code.

Morphological identification: Morphological identification was done as described by Kirk & Cooper (2009).

Molecular identification

DNA barcoding studies: The obtained ITS sequence of *Aspergillus niger* (MBL-1511) was used to extract the ITS1 and ITS2 sub region with ITSx online tool (Bengtsson-Palme *et al*., 2013). The ITS sequences of different Ascomycota members were obtained from NCBI public database of nucleotides. The selection criteria of sequences was based on the length of fungal sequence (>700 bps) from 400 search results. The selected

sequences were aligned with CLustalW2 (Multiple sequence alignment program) available online on Expasy (http://www.expasy.org/genomics). The obtained alignments were used for the construction of a phylogeny tree with ClustalW2-Phylogeny (online tool of EMBL-EBI) with neighbor-joining matrix. The morphologically identified fungal hyper lipase producer was exploited for extraction of gDNA (genomic DNA) as described earlier (van Kan *et al*., 1991). 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/).

Physical mutagenesis: Gamma irradiation was used for enhanced lipolytic potential of the fungal hyper producer.

Strain improvement by Gamma irradiation treatment: The hyper producer of *A. niger* (MBL-1511) containing, spore suspension (5 mL) was treated with gamma irradiation. Cell suspension (5 mL) was transferred in each vial, sealed with plastic cover and parafilm. These vials were exposed to gamma irradiator. Gamma radiation with different doses were selected as, 10, 20, 40, 60, 80, 100, 120, 140 and 160 Gy. These vessels were subjected to the γ-irradiation [Gamma cell 3000, Canada imported (Cs^{137}) 30 year half-life dose 660 Gr/h] at Nuclear Institute of Agricultural and Biology (NIAB), Faisalabad for enhancement of lipases production. After different time intervals, the spore suspension of treated radiation (0.1 mL) was maintained on the (4%) PDA plates. Oxgall (1%) as colony restrictor was added to each petri plate containing (4%) PDA, treated spore suspension and incubated for 3-5 days at 30ºC. The kill curve was prepared and time of exposure was optimized for the mutation of potent producer for hyper production of lipases.

Chemical mutagenesis: Chemical mutagenesis for enhance lipases production was carried out using various mutagens *i.e.,* nitrous acid, sodium azide and ethyl methane sulphonate (EMS).

Strain improvement by Nitrous acid mutagenesis: Cell culture of *A. niger* (MBL-1511) prepared in Vogel's medium (g/L KH_2PO_4 0.5, NH_4NO_3 0.2, $(NH_4)_2SO_4$ 0.4, $MgSO_4.7H_2O$ 0.02, Peptone 0.1, Trisodium citrate 0.5, Yeast extract 0.2, Glucose 50% (w/v) and pH 5.5) was subjected to nitrous acid (0.1M sodium nitrite in phosphate buffer, pH 5.0) treatment at interval of 30, 60, 90, 120, 150 and 180 min by incubating the mixture at 30°C. Treated cells were washed thrice at 10,000 rpm to remove the traces of mutagen. After suspending the cells in saline, these were placed on PDA plates having 1% oxgall and placed at 30°C for 3-5 days for the preparation of kill curve. After growth, mutants forming larger zones were picked up and transferred to PDA slants and were further tested quantitatively (Rajeshkumar & Ilyas, 2011). Further screening and selection of mutants was performed as reported by (Iftikhar *et al*., 2010).

Strain improvement by Sodium azide mutagenesis: Chemical mutagenesis for the strain improvement was performed using sodium azide. Spore suspensions of *A. niger* (MBL-1511) was prepared by using phosphate buffer (pH 7.0) Elliaiah *et al.* (2002). Then it was subjected to mutagenic treatment by adding 1 mL of sterile solution of sodium azide (250µg mL^{-1} in phosphate buffer) to spore suspension (9 mL). The reaction was allowed to proceed. Control tubes were run without any chemical mutagen. Time interval of mutagenic treatment ranged from 30 to 150 min with interval of 30 min and was placed in incubator at 30ºC. Using sterilized distilled water, hyper producer was washed thrice at 5000 rpm for 10 min and again re-suspended in sterilized buffer (10 mL). The samples were serially diluted with the same buffer and plated on Sabouraud's Dextrose agar medium as it inhibited bacterial growth (Bapiraju *et al*., 2004). Further screening and selection of mutants was performed as reported by (Iftikhar *et al*., 2010).

Strain improvement by EMS mutagenesis: Chemical mutagenesis for the strain improvement was performed using EMS. Spore suspensions of *A. niger* (MBL-1511) was prepared by using phosphate buffer pH 7.0. Ellaiah *et al*. (2002). Then it was subjected to mutagenic treatment by adding 1 mL of sterile solution of sodium azide (250µg mL^{-1} in phosphate buffer) to spore suspension (9 mL). The reaction was allowed to proceed. Control tubes were also kept without any chemical mutagen. Time interval of mutagenic treatment ranged from 30 to 150 min with interval of 30 min and was placed in incubator at 30ºC. Using sterilized distilled water, hyper producer was washed thrice at 5000 rpm for 10 min and again re-suspended in 10mL sterilized buffer. The samples were serially diluted with the same buffer and plated on Sabouraud's Dextrose agar medium as it inhibits bacterial growth (Bapiraju *et al*., 2004; Rajeshkumar & Ilyas, 2011). Further screening and selection of mutants was performed as reported by (Iftikhar *et al*., 2010).

Proximate analysis: In the present study, eight different meals *i.e.,* soybean, coconut, sunflower, rice bran, sesame, almond, rice husk and brassica were used for enhanced lipases production. Before optimization different analysis were performed to in order to check the nutrients composition (moisture, ash, fat, fiber and protein) % of the meals before and after fermentation by wild (MBL-1511) and mutant MBL-1511^{SA-4}(150 min) strains of *A. niger*. Meals were analyzed on dry weight basis for crude protein (Method No. 46-30), crude fat (Method No. 30-25), crude fiber (Method No. 32-10), ash (Method No. 08-01) and moisture (Method No. 44-15A), according to their respective procedures described in (Anon., 2000).

Production of extracellular lipases: Fungal strains were subjected to solid state fermentation method for lipase production as reported earlier (Iftikhar *et al*., 2010). The procedure was devised for the extraction of lipases Kempka *et al*. (2008).

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

Results

Isolation and screening of fungal strains: One hundred and forty (140) samples of fungi were isolated from different environment and food samples such as air, bread, butter, chicken roasted in oil, decaying fruits, decaying vegetables, fish roasted in oil, fry potato, pickle, pizza and soil by serial dilution method. The fungal isolates were screened quantitatively through solid state fermentation (SSF) and results were shown (Fig. 1-12). Among all fungal isolates, cultural isolate MBL-1511 obtained from chicken roasted in oil gave the maximum production with lipase activity 5.3±0.04[a] IU/ML (Fig. 1). Therefore, cultural isolate MBL-1511 was selected for further studies.

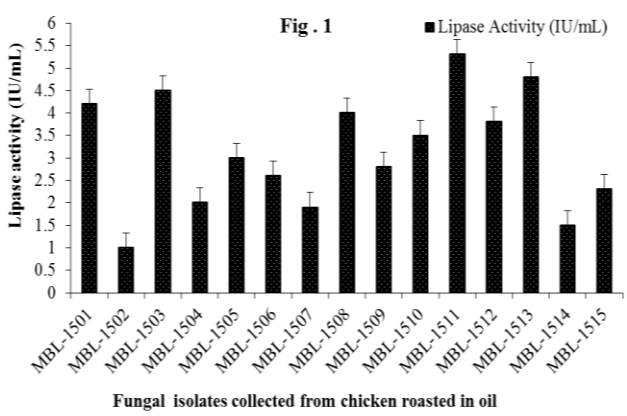
Morphological identification of hyper producer fungal strain: Selected hyper producer was studied under microscope (Fig. 13). It resembled with *Aspergillus niger* due to color of spores, shape of vesicles, morphology of the hyphae, conidial width and mycelium length.

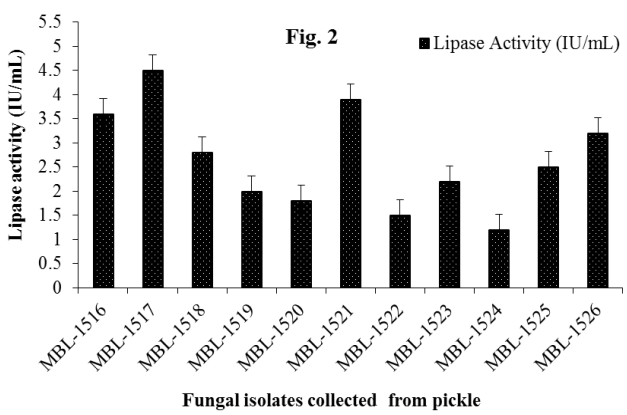
Molecular studies of hyper-producer: To identify the fungi on genetic basis, 18S rRNA gene sequence was approximately used as a molecular marker (Fig. 14). The entire mass of mycelium from the colony was used to extract gDNA. The two universal primers ITS1 and ITS4 were used. Obtained sequence was stored in gene bank with the accession no. [GenBank: KP172477]. The sequence analysis suggested its highest similarities with *Aspergillus niger* (Fig. 15).

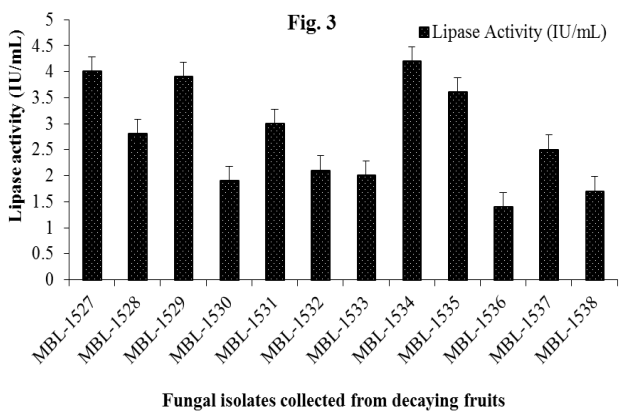
Mutagenesis treatment and identification of hyperproducer: It is well-known that chemical and physical mutation has great impact on the lipases production. The purified hyper producer of *Aspergillus niger* (MBL-1511) was subjected to various physical (gamma irradiation) and chemical mutagenesis (nitrous acid, sodium azide and EMS). From kill curves (Figs. 16-19) it was observed that 147.27% extracellular lipases production was increased after 150 min of the mutagenic treatment with sodium azide (Table 1). The selected mutant was assigned the code MBL-1511^{SA-4}(150 min) and was used for further studies in parallel with wild (MBL-1511) strain.

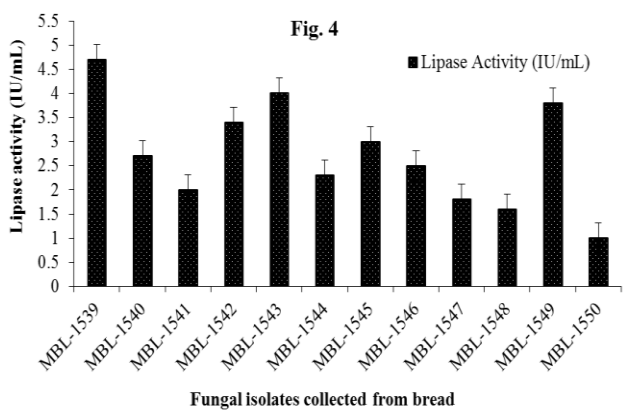
Conditions of fermentation: Each value is mean of triplicate and ± denote the standard error value. *Incubation temperature 30°C, Incubation period 48 h and pH 7.0.

Proximate analysis: Eight different agro-industrial byproducts such as sesame meal, rice husk, soybean meal, coconut meal, sunflower meal, almond meal, brassica meal and wheat bran were tested to see the ingredients composition before and after the fermentation by wild (MBL-1511) and mutant MBL-1511^{SA-4}(150 min) strains of *A. niger* through SSF (Table 2). Proximate analysis of brassica meal showed maximum protein 43.6 % before fermentation, 45.8% and 49.5% after fermentation by wild and mutant strain respectively through SSF. The results showed that maximum fats found in brassica meal before fermentation was 9.73% and after fermentation the same were reduced to 7.26% and 6.31% for wild (MBL-1511) and mutant MBL-1511^{SA-4}(150 min) strains of *A. niger* respectively. Increase in the crude fiber contents were also observed after fermentation in wild and mutant fermented meals of brassica (Table 2). Therefore brassica meal was used for further studies.

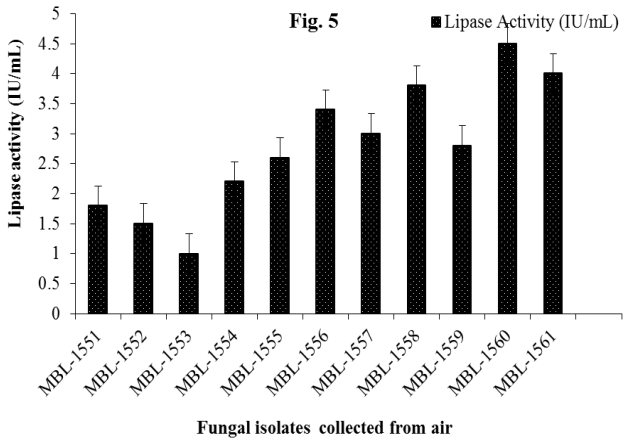


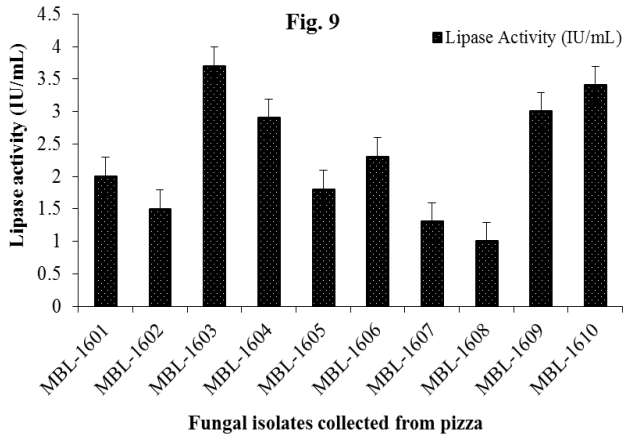


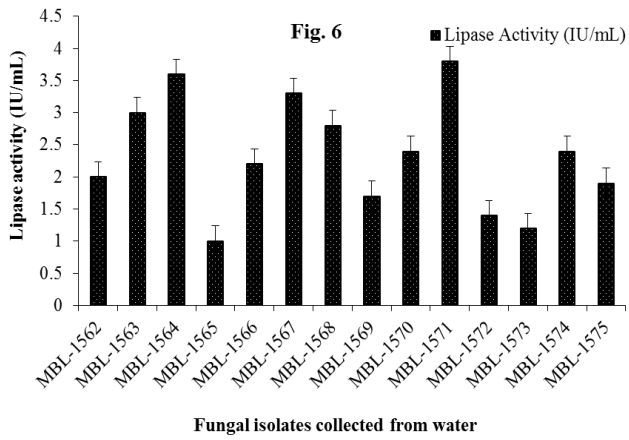


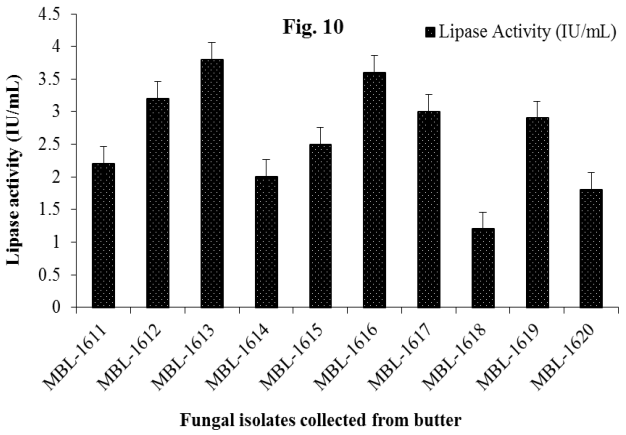


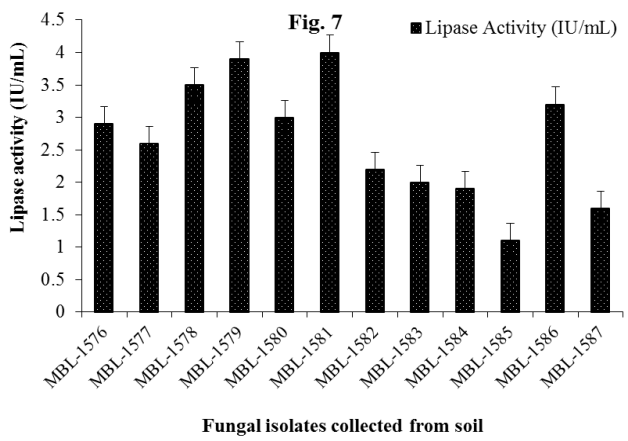
Figs. 1-4. Screening of various fungal isolates (fig. 1) chicken roasted in oil, (fig. 2) pickle, (fig. 3) decaying fruits, and (fig. 4) bread collected from indigenous source through solid state fermentation techinque (SSF). Error bars show standard deviation among three observations.

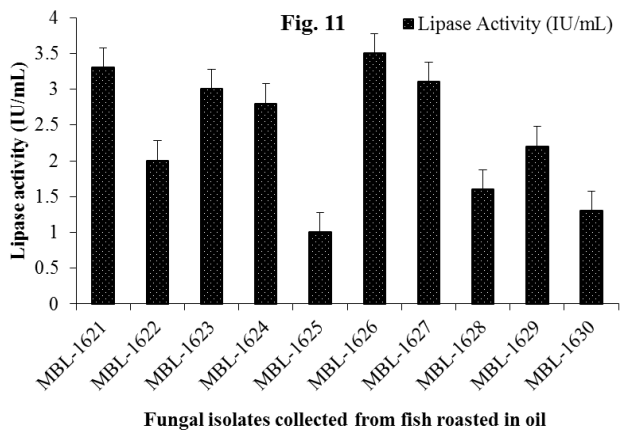


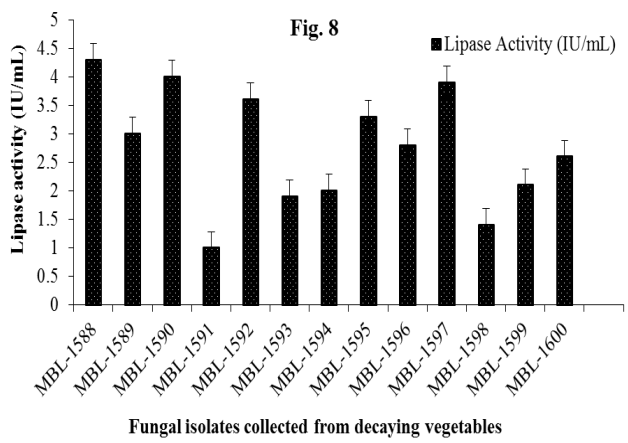


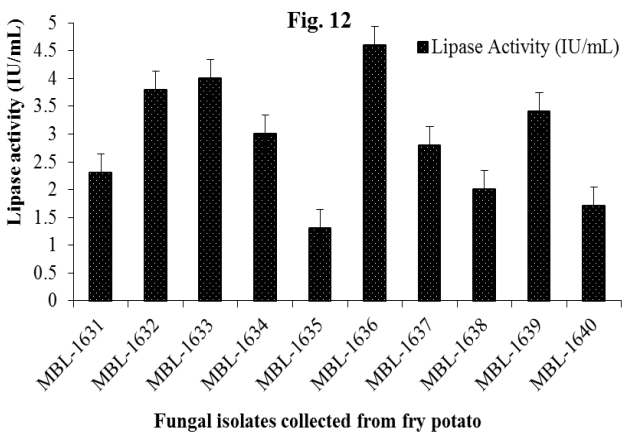












Figs. 5-12. Screening of various fungal isolates (fig. 5) air, (fig. 6) water, (fig. 7) soil, (fig. 8) decaying vegetables, (fig. 9) pizza, (fig. 10) butter, (fig. 11) fish roasted in oil and (fig. 12) fry potato collected from indigenous source through solid state fermentation techinque (SSF). Error bars show standard deviation among three observations.

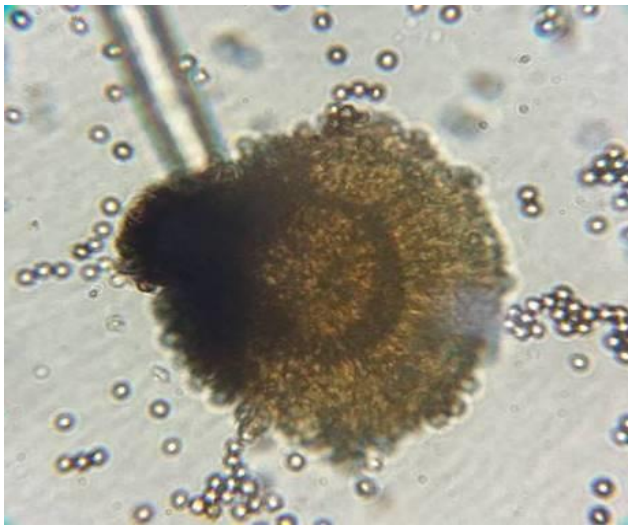


Fig. 13. The fungal hyper producer culture was examined 100X under the microscope.

Optimization of other cultural conditions for the production of extracellular lipases

Effect of agro-industrial by-products: Fungi possess differential lipases production potential towards different agro-industrial by products (Fadiloglu & Erkmen, 2002). The next step was to select an appropriate substrate for the maximized growth of *A. niger* (MBL-1511) and subsequent lipases production. For this purpose, earlier mentioned agro-industrial by products were exploited for extracellular lipases production by wild (MBL-1511) and mutant MBL-1511^{SA-4}(150 min) of *A. niger* through SSF (Fig. 20). Among all the tested meals, brassica meal gave significantly highest extracellular enzyme activity *i.e.*, 10.67±0.01^{a} IU/mL (wild) and 19.58±0.04^{a} IU/mL (mutant) respectively. There was also an increase in the specific activity of the enzyme both by wild and mutant strains of the fungus, soybean meal gave maximum specific activity by wild (25.79 IU/mg) and mutant (41.65 IU/mg) (Fig. 20). Thus, brassica meal was found to be the best source of carbon and nitrogen for enhanced lipase production and was used for further studies.

Effect of substrate concentration: Substrate concentration plays key role in lipases production. Various concentration of brassica meal was tested *i.e.*, (5, 10, 15, 20, 25, 30 & 35) g to optimize the lipolytic potential of wild (MBL-1511) and mutant MBL-1511^{SA-4}(150 min) strains of *A. niger* through SSF. Among all tested substrates, concentration of 10g of substrate gave the highest units of extracellular lipases by 10.71±1.1^{a} IU/mL (wild) and 19.65±0.55^{a} IU/mL (mutant) strain (Fig. 21). On the other hand 10g substrate also gave maximum specific activity in both cases. Therefore 10g substrate gave the maximum extracellular units so; brassica meal (10g) was used for further studies.

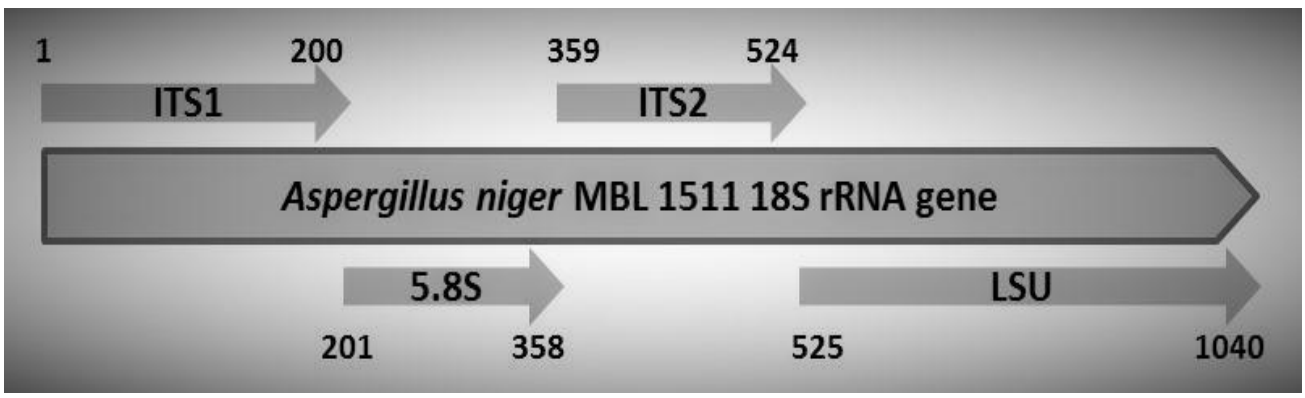


Fig. 14. The diagrammatic representation of ITS regions of 18S rRNA of *Aspergillus niger* (MBL-1511).

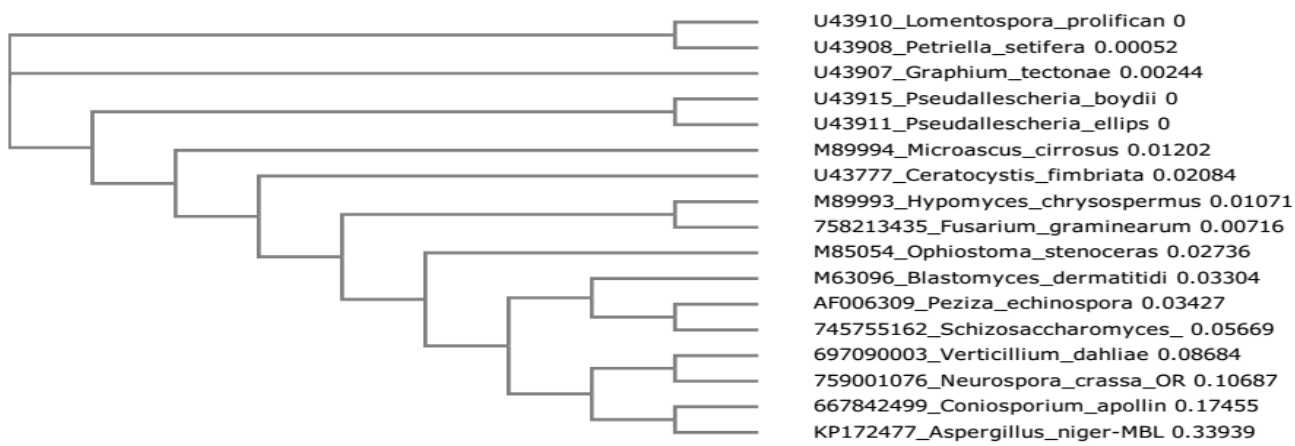
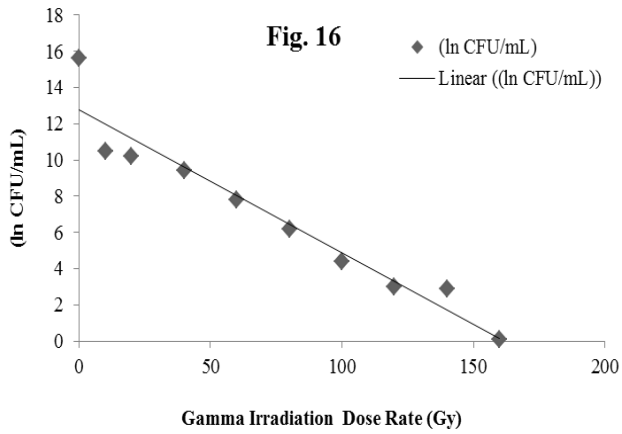
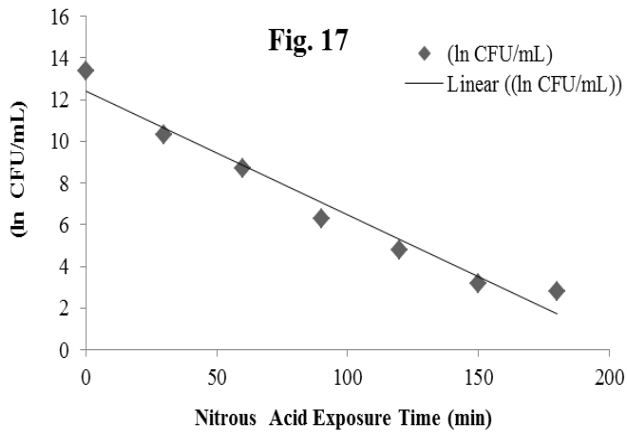
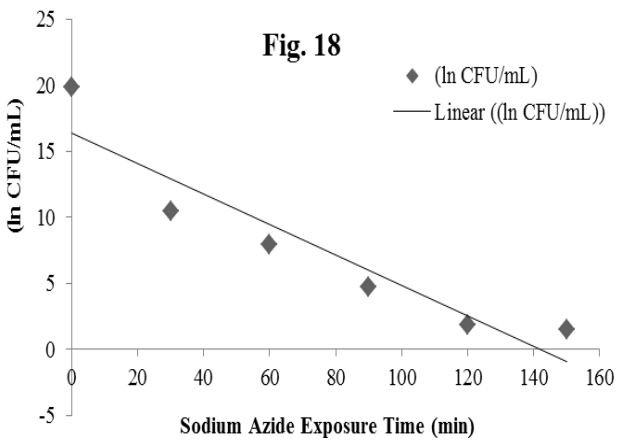


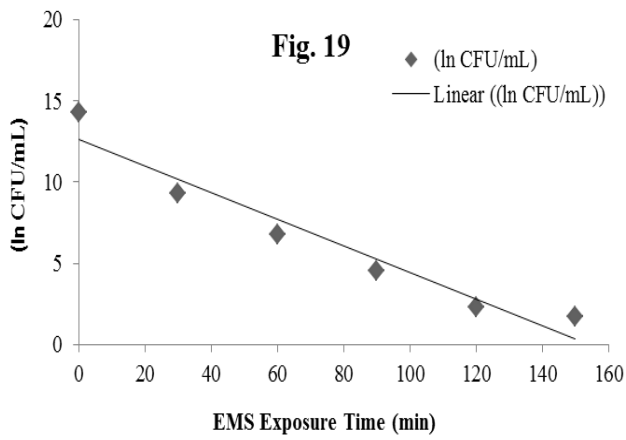
Fig. 15. The phylogenetic comparison (Neighbor-joining with default values of ClustalW2) of ITS sequence of *Aspergillus niger* (KP172477) and members of Ascomycota available on NCBI public data base (ITS sequence >700 bps). Each sequence shows its corresponding genbank identification (gi) following with fungal species, strain and scoring matrix values.





Figs. 16-17. Dose Kill curve after mutagenic dose of (fig. 16) Gamma irradiation treatment, Survival%= 2.9/15.6*100=18.58, Kill%=100-18.42=81.42% and (fig. 17) Nitrous acid treatment, Survival%= 2.9/13.4*100=21.64%, Kill %=100-21.64= 78.36%.





Figs. 18-19. Dose Kill curve after mutagenic dose of (fig. 18) Sodium azide treatment, Survival %=1.5/19.9*100= 7.53%, Kill %= 100-7.53= 92.47%, (fig. 19) EMS treatment, Survival %=1.7/14.3*100=11.88% and Kill %= 100-11.88=88.12%.

Effect of type of inoculum: Type of Inoculum also plays an important role for the production of extracellular lipases. In the present studies effect of different types of inoculum such as vegetative and spore inoculum was checked for production of extracellular lipases. Spore inoculum gave the maximum production of lipases both in case of wild (10.75±0.45[a] IU/mL) and mutant (20.64±0.66[a] IU/mL) strains of *A. niger* (Fig. 22). Hence, spore inoculum was selected for further studies.

Effect of size of inoculum: Size of inoculum also has great influence on the production of lipases. Different inoculum size ranging from 0.5-5 mL with an interval of 0.5 mL were tested for extracellular lipases production by wild (MBL-1511) and mutant MBL-1511^{SA-4}(150 min) strains of *A. niger* through SSF (Fig. 23). The highest extracellular output of enzyme was obtained by wild (10.83±0.31[a] IU/mL) and mutant (20.66±0.30[a] IU/mL) and mutant, when 1.0 mL level of inoculum was used.

Effect of moistening agents: Moistening agents (diluents) also play an important role in the production of lipases. Type of extractant influenced extracellular lipases production by wild (MBL-1511) and mutant MBL-1511^{SA-4}(150 min) strains of *A. niger*. Phosphate buffer (pH 6.8) as an extractant supported the highest lipases production both by wild (11.85±0.10[a] IU/mL) and mutant (21.76±0.11[a] IU/mL) strains of *A. niger* as shown in (Fig. 24). Therefore Phosphate buffer (pH 6.8) as an extractant was selected for further studies.

Effect of volume of the diluent: Volume of diluent, also plays an important role in the production of lipases by wild (MBL-1511) and mutant MBL-1511^{SA-4}(150 min) strains of *A. niger* through SSF. In the present study, different ranges of diluents were tested *i.e.,* 5-30mL with the intervals of 5mL by wild and mutant strains of *A. niger* (MBL-1511). As far as the volume of the diluent is concerned there was 10mL of diluent supported maximized production of enzyme both by wild (12.98±0.30[a] IU/mL) and mutant (21.95±0.50[a] IU/mL) and mutant strains (Fig. 25).

Effect of initial pH: Initial pH of the diluent also has a great role in lipases production. In the present study different pH of diluent was tested *i.e.,* 5.8-7.8 pH with the interval of 0.4 pH that influenced extracellular lipases production by wild and mutant strains of *A. niger* by SSF. Regarding pH of diluent, it was observed that pH 6.2 supported maximized enzyme production by wild (13.66±0.88[a] IU/mL) and mutant (23.33±0.34[a] IU/mL) strains of *A. niger* by SSF (Fig. 26).

Table 1. Activity of extracellular lipase produced by various potent mutants of *Aspergillus niger* (MBL-1511) using solid state fermentation technique.

Wild/mutant	Zone size (mm)	Lipase activity (IU/mL)	% Increase or decrease in activity
	Strain improvement by gamma irradiation treatment		
Wild	6	5.5±.32[a]	100
Mutants			
MBL$^{\text{Gamma-1}}_{\text{(140 Gy)}}$	10	5.72 ± 0.12[f]	104
MBL$^{\text{Gamma-2}}_{\text{(140 Gy)}}$	11	5.84 ± 0.04[e]	106.18
MBL$^{\text{Gamma-3}}_{\text{(140 Gy)}}$	12	5.90 ± 0.08[c]	107.27
MBL$^{\text{Gamma-4}}_{\text{(140 Gy)}}$	11	6.00 ± 0.1[b]	109.09
MBL$^{\text{Gamma-5}}_{\text{(140 Gy)}}$	7	4.52 ± 0.12[j]	82.18
MBL$^{\text{Gamma-6}}_{\text{(140 Gy)}}$	13	5.92 ± 0.09[d]	107.63
MBL$^{\text{Gamma-7}}_{\text{(140 Gy)}}$	14	6.12 ± 2.37[a]	111.27
MBL$^{\text{Gamma-8}}_{\text{(140 Gy)}}$	10	4.87 ± 0.44[h]	z88.54
MBL$^{\text{Gamma-9}}_{\text{(140 Gy)}}$	9	4.74 ± 0.32[i]	86.18
MBL$^{\text{Gamma-10}}_{\text{(140 Gy)}}$	8	5.22 ± 0.12[g]	94.90
	Strain improvement by nitrous acid treatment		
Mutant			
MBL$^{\text{NA-1}}_{\text{(180 min)}}$	15	4.45 ± 0.44[i]	80.90
MBL$^{\text{NA-2}}_{\text{(180 min)}}$	13	4.62 ± 0.1[h]	84.00
MBL$^{\text{NA-3}}_{\text{(180 min)}}$	8	5.84 ± 0.53[a]	106.18
MBL$^{\text{NA-4}}_{\text{(180 min)}}$	9	5.53 ± 0.03[f]	100.54
MBL$^{\text{NA-5}}_{\text{(180 min)}}$	10	5.62 ± 0.07[c]	102.18
MBL$^{\text{NA-6}}_{\text{(180 min)}}$	12	5.74 ± 0.14[b]	104.36
MBL$^{\text{NA-7}}_{\text{(180 min)}}$	11	5.35±0.35[d]	97.27
MBL$^{\text{NA-8}}_{\text{(180 min)}}$	7	4.92 ± 0.02[g]	89.45
MBL$^{\text{NA-9}}_{\text{(180 min)}}$	14	5.12 ± 0.03[e]	93.09
MBL$^{\text{NA-10}}_{\text{(180 min)}}$	12	4.80 ± 0.42[j]	87.27
	Strain improvement by sodium azide treatment		
Mutants			
MBL$^{\text{SA-1}}_{\text{(150 min)}}$	12	6.70 ± 0.37[d]	121.81
MBL$^{\text{SA-2}}_{\text{(150 min)}}$	10	6.60 ± 0.31[e]	120
MBL$^{\text{SA-3}}_{\text{(150 min)}}$	9	7.00 ± 1.00[b]	127.27
MBL$^{\text{SA-4}}_{\text{(150 min)}}$	14	8.10 ± 1.05[a]	147.27
MBL$^{\text{SA-5}}_{\text{(150 min)}}$	11	6.51 ± 0.1[f]	118.36
MBL$^{\text{SA-6}}_{\text{(150 min)}}$	13	6.95 ± 0.05[c]	126.36
MBL$^{\text{SA-7}}_{\text{(150 min)}}$	8	5.94 ± 0.02[g]	108
MBL$^{\text{SA-8}}_{\text{(150 min)}}$	9	5.75 ± 0.2[h]	104.54
MBL$^{\text{SA-9}}_{\text{(150 min)}}$	13	5.0 ± 0.5[i]	90.90
MBL$^{\text{SA-10}}_{\text{(150min)}}$	10	4.80 ± 0.42[j]	87.27
	Strain improvement by EMS treatment		
Mutants			
MBL$^{\text{EMS-1}}_{\text{(150 min)}}$	11	5.10 ± 0.03[h]	92.72
MBL$^{\text{EMS-2}}_{\text{(150 min)}}$	17	6.22 ± 0.03[b]	113.09
MBL$^{\text{EMS-3}}_{\text{(150 min)}}$	11	4.44 ± 0.03[j]	80.72
MBL$^{\text{EMS-4}}_{\text{(150 min)}}$	10	5.32 ± 0.03[g]	96.72
MBL$^{\text{EMS-5}}_{\text{(150 min)}}$	14	6.12 ± 0.03[c]	111.27
MBL$^{\text{EMS-6}}_{\text{(150 min)}}$	15	5.65 ± 0.03[f]	102.72
MBL$^{\text{EMS-7}}_{\text{(150 min)}}$	16	6.42 ± 0.03[a]	116.72
MBL$^{\text{EMS-8}}_{\text{(150 min)}}$	13	5.84 ± 0.03[d]	106.18
MBL$^{\text{EMS-9}}_{\text{(150 min)}}$	12	4.98 ± 0.03[i]	90.54
MBL$^{\text{EMS-10}}_{\text{(150 min)}}$	9	5.72 ± 0.03[e]	104.0

Effect of incubation temperature: Incubation temperature shows dynamic role in lipases production. In the present study different levels of incubation temperature were tested *i.e.*, 20-55ºC with an interval of 5ºC through SSF. Incubation temperature of 30ºC supported maximized lipases production both from wild (13.75±0.91[a] IU/mL) and mutant (23.81±0.19[a] IU/mL) strains of *A. niger* by SSF (Fig. 27). Therefore, incubation temperature of 30°C was used for further studies.

Effect of rate of fermentation: Rate of fermentation plays vital role in lipases production. Different incubation periods ranges from 12 to 144 hours with an intervals of 12 h were tested for the lipases biosynthesis by wild (MBL-1511) and mutant MBL-1511$^{\text{SA-4}}$(150 min) strains of *A. niger* through SSF. Present findings reported maximized production after 72 h of incubation both in wild (15.5±0.15[a] IU/mL) and (25.66±0.31[a] IU/mL) mutant cases (Fig. 28). Therefore Incubation period of 72 h was optimized for further studies.

Effect of additional organic carbon sources: Additives to the basal substrate are very influential towards lipases production. Organic carbon sources including glucose, lactose, maltose, sucrose, fructose, tween 80, dextrose and starch at 1% (w/v) were checked for the biosynthesis of extracellular lipases by wild (MBL-1511) and mutant MBL-1511$^{\text{SA-4}}$(150 min) strains of *A. niger* through SSF. Tween 80 (1% w/v) gave significantly highest wild extracellular enzyme activity (17.8±0.35[a] IU/mL) and mutant (27.8±0.2[a] IU/mL) strains of *A. niger* (Fig. 29). Therefore tween 80 @ 1% (w/v) was optimized for further studies.

Effect of additional organic and inorganic nitrogen sources: Organic and inorganic nitrogen sources have great influence on the fungal lipases production. Different organic *i.e.,* Urea, peptone, casein, yeast extract, nutrient broth and inorganic nitrogen sources as malt extract, sodium nitrate, ammonium acetate, ammonium nitrate and ammonium chloride were used to check the lipolytic potential for lipases production by wild (MBL-1511) and mutant MBL-1511$^{\text{SA-4}}$(150 min) strains of *A. niger* through SSF. Yeast extract acted as the best organic nitrogen source with enzyme activity 18.5±0.5[a] IU/mL (wild) and 30.32 ±0.7[a] IU/mL (mutant), while on the other hand yeast extract also gave the maximum specific activity by wild (37.75 IU/mg) and mutant (54.14 IU/mg) case. Therefore yeast @ 1% (w/v) extract was optimized for further studies (Fig. 30).

Effect of additional oils as organic carbon sources: Organic oils as carbon sources also has pivotal role in the production of lipases. Organic carbon sources including, cotton seed oil, olive oil, brassica oil, almond oil, coconut oil, sunflower oil and soybean oil, canola oil, sesame oil, sunflower oil and caster oil at 1% (v/v) were checked for the production of extracellular lipases by wild (MBL-1511) and mutant MBL-1511$^{\text{SA-4}}$(150 min) strains of *A. niger* through SSF. In the present studies olive oil gave significantly highest extracellular enzyme activity both by wild extracellular lipases *i.e.,* 20.21±0.06[a] IU/mL (wild) and 32.5±0.02[a] IU/mL (mutant). On the other hand coconut gave maximum specific activity *i.e.,* (40.54 IU/mg) by wild and (58.69 IU/mg) by mutant. Finally, a potent mutant of *A. niger* [MBL-1511$^{\text{SA-4}}$(150 min)] with an increased activity of 161 % over the wild strain was obtained when olive oil was used at 1% (v/v) concentration (Fig. 31).

Table 2. Proximate Analysis of agro-industrial byproducts before and after the fermentation by wild (MBL-1511) and mutant (MBL-1511^{SA-4}(150 min) strains of *Aspergillus niger* through Solid state fermentation.

Sr #	Substrate before and after fermentation by wild and mutant strains	Substrate (g)	Moisture (%)	Fat (%)	Ash (%)	Fiber (%)	Protein (%)
	Unfermented meal		9.7	5.33	11.5	6.8	18.7
1.	Wild fermented meal	Sesame meal	8.7	5.1	10.4	7.5	20.32
	Mutant fermented meal		8.1	4.72	9.42	8.1	21.5
	Unfermented meal		6.8	2.36	15.4	14.53	9.12
2.	Wild fermented meal	Rice Husk	7.1	2.1	12.3	15.6	11.4
	Mutant fermented meal		7.4	1.98	11.4	16.7	14.5
	Unfermented meal		7.7	6.42	5.76	6.42	41.4
3.	Wild fermented meal	Soybean meal	8.3	5.94	5.21	7.1	43.2
	Mutant fermented meal		8.5	5.12	4.92	7.8	44.1
	Unfermented meal		7.73	9.73	6.72	20.6	43.6
4.	Wild fermented meal	Brassica meal	8.65	7.26	7.3	21.7	45.8
	Mutant fermented meal		8.84	6.31	8.1	22.4	49.5
	Unfermented meal		8.8	5.88	6.25	19.15	17.5
5.	Wild fermented meal	Sunflower meal	7.9	4.51	5.94	20.2	19.5
	Mutant fermented meal		6.8	3.98	5.44	21.43	20.2
	Unfermented meal		7.8	5.81	5.25	16.32	26.2
6.	Wild fermented meal	Almond meal	8.3	4.82	4.34	18.72	27.3
	Mutant fermented meal		8.7	3.76	3.97	19.45	29.4
	Unfermented meal		8.5	5.63	6.45	12.01	38.2
7.	Wild fermented meal	Coconut meal	9.6	5.1	5.87	13.4	39.4
	Mutant fermented meal		9.9	4.8	5.21	14.5	40.5
	Unfermented meal		2.5	3.28	3.8	7.53	17.5
8.	Wild fermented meal	Wheat bran	3.5	2.92	3.5	8.54	19.2
	Mutant fermented meal		3.9	2.76	3.1	7.91	19.9

Each value is mean of triplicate values

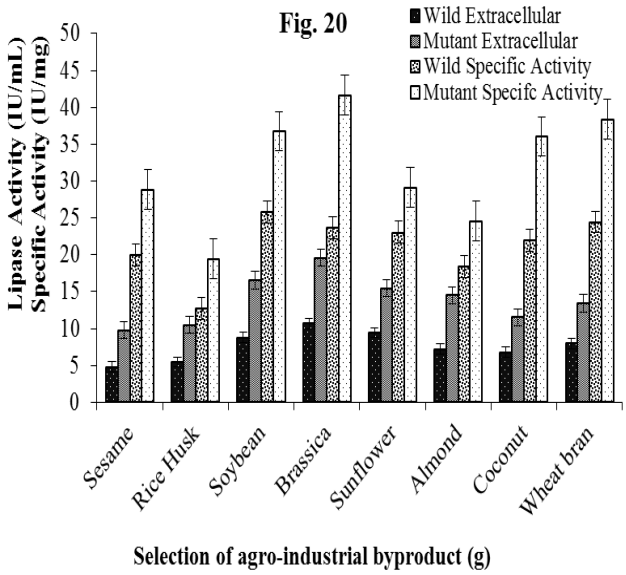
Discussion

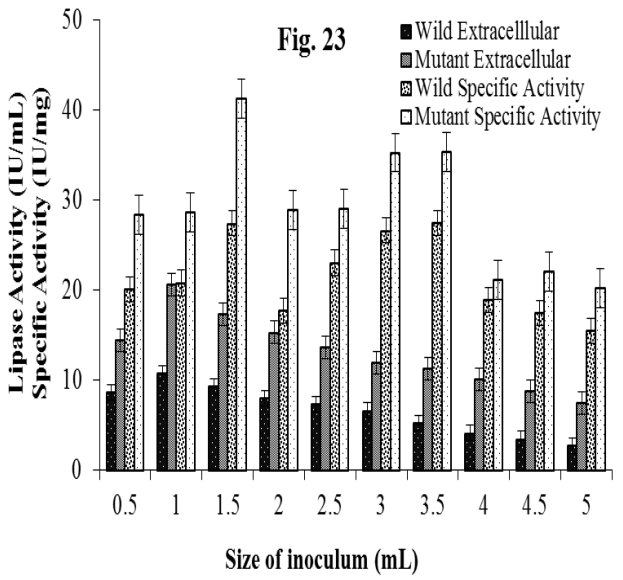
Isolation and screening of lipolytic fungi: Out of one hundred and forty (140) fungal isolates, cultural isolate MBL-1511 obtained from chicken roasted in oil gave the maximum production while other cultures did not show the major production it might be due to the reason that enzyme production was linked with the cell growth (Gutarra *et al.,* 2007). Therefore, it was selected for further studies.

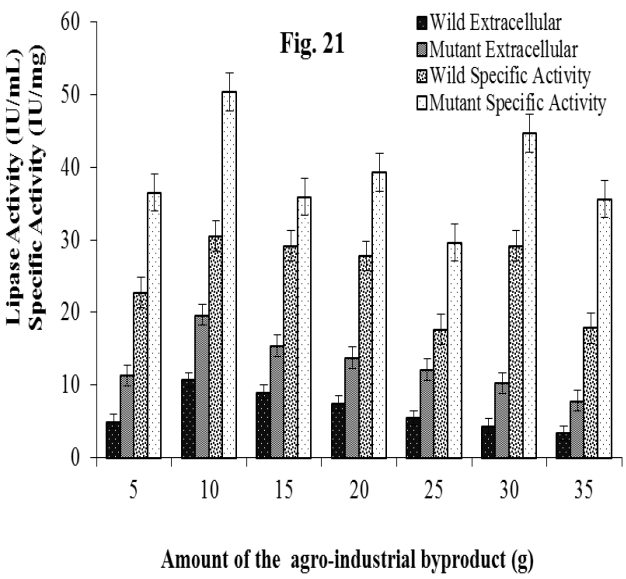
Morphological Identification of hyper producer fungal strain: Selected hyper producer was studied under microscope. It was identified as a member of Ascomycota named "*Aspergillus niger"* that was confirmed by Index of fungorum and Doctor fungus; www.doctorfungus.org. It resembled with *Aspergillus niger* due to color of spores, shape of vesicles, morphology of the hyphae, conidial width and mycelium length as earlier reported by (Al-Hindi *et al*., 2011) for lipases production.

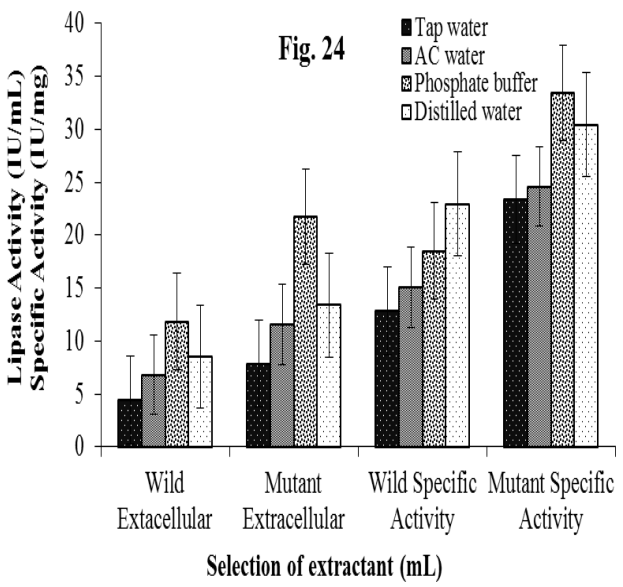
Identification of hyper producer on genetic basis: To identify the fungi on genetic basis, 18S rRNA gene sequence is being widely utilized as a molecular marker. The whole mass of mycelium from the colony was used to extract gDNA. For sequence based identification, 18S rRNA gene was targeted. The two universal primers ITS1 and ITS4 were used as reported earlier (Iftikhar *et al*., 2014; Bashir *et al*., 2017). Pereira *et al*. (2014) also reported work on the fungal strain identification by using technique DNA sequening for lipases production. The sequence analysis suggested its highest similarity with *Aspergillus niger*. Obtained sequence was deposited in genbank with the accession no. KP172477.

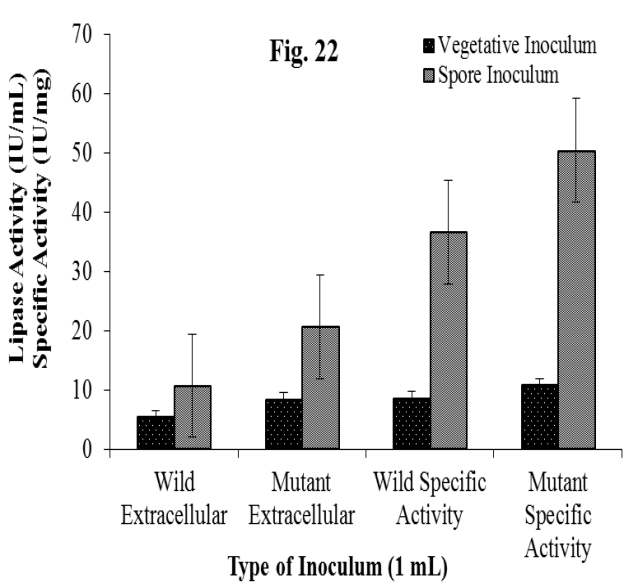
Mutagenic treatment and identification of hyperproducer: It is well-known that chemical and physical mutation has great impact on the lipases production. Similar findings are reported in earlier studies (Bapiraju *et al*., 2004). UV and chemically treated fungal strains of *Rhizopus* sp. showed 133% to 232% higher production of lipases than the wild strains. The sodium azide mutant produced 133% higher phosphate solubilization than the wild strain. The mutant strains of *Aspergillus niger* and *Aspergillus fumigatus* by sodium azide mutagenesis increased two folds enzyme production over wild strain and *Penicillium* sp. by sodium azide showed 1.5 folds increased enzyme production over wild strain (Rajeshkumar & Ilyas, 2011). In another study, mutant strains of *Aspergillus niger* showed five to seven fold enhanced productivity of lipase over the wild strain (Mahadik *et al*., 2004). The selected mutant was assigned the code MBL-1511^{SA-4}(150 min) and used for further studies in parallel with wild strain.

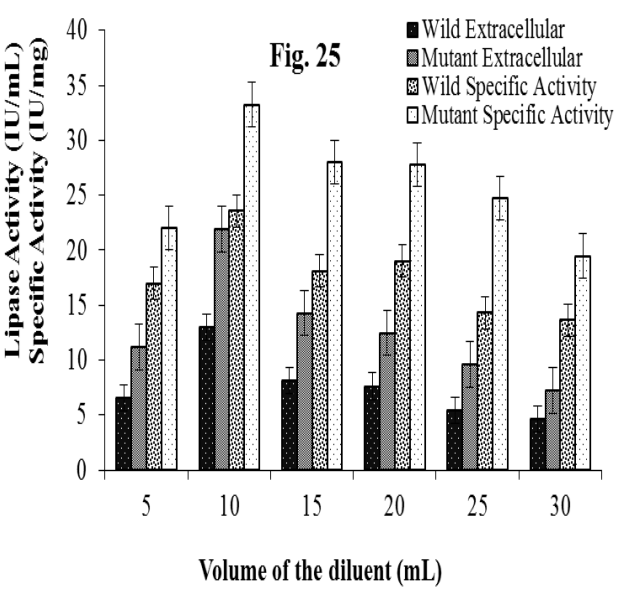




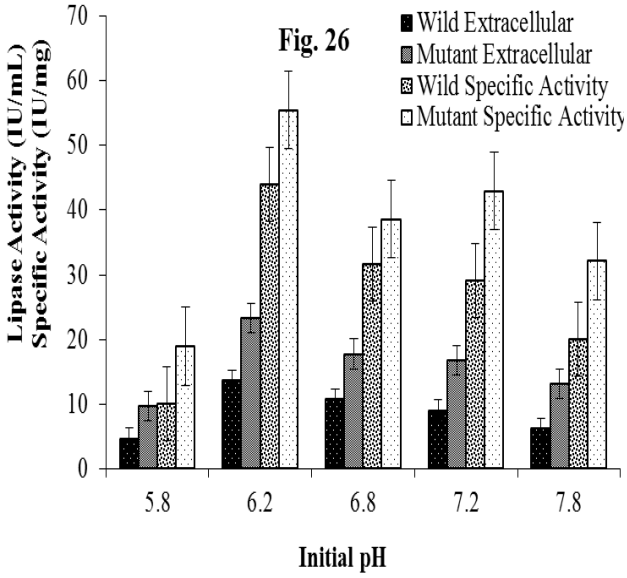


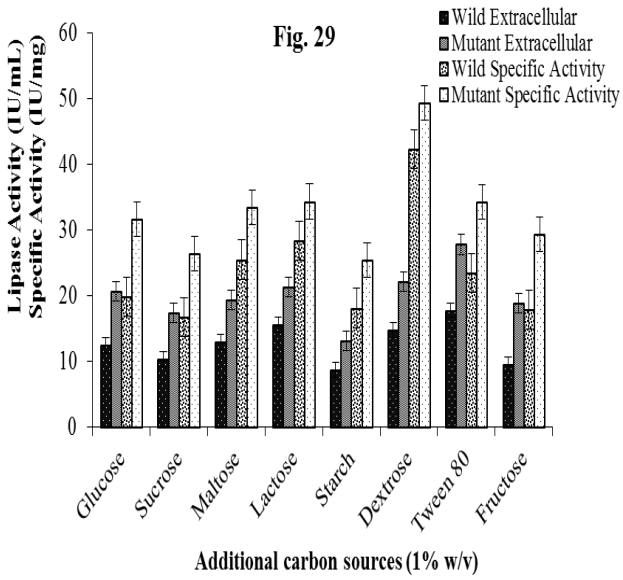


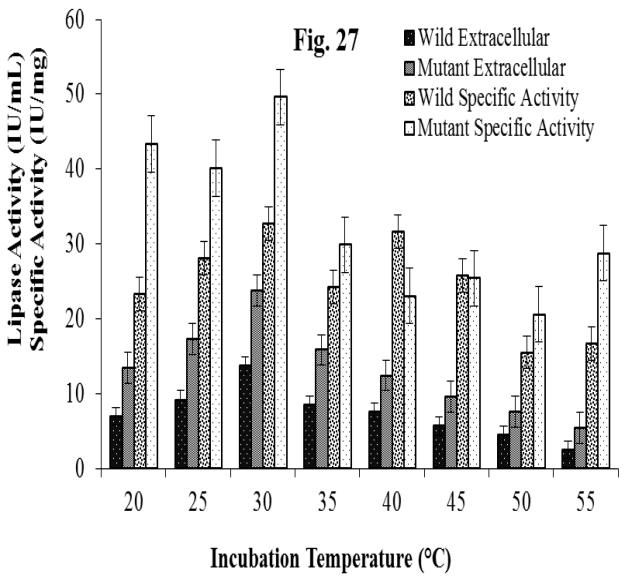


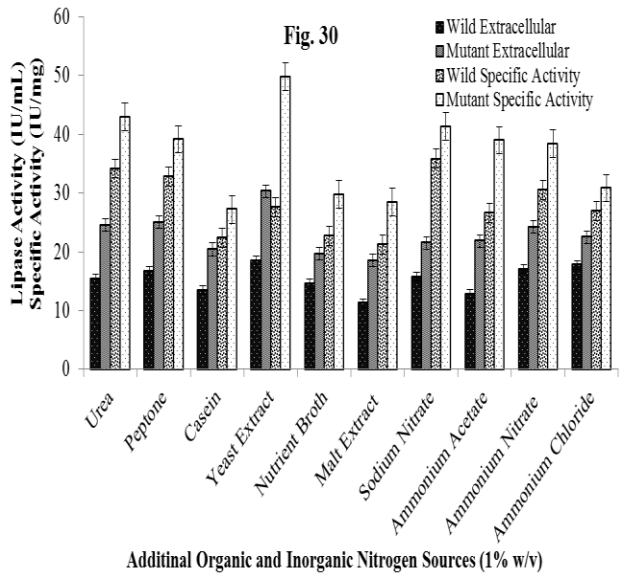


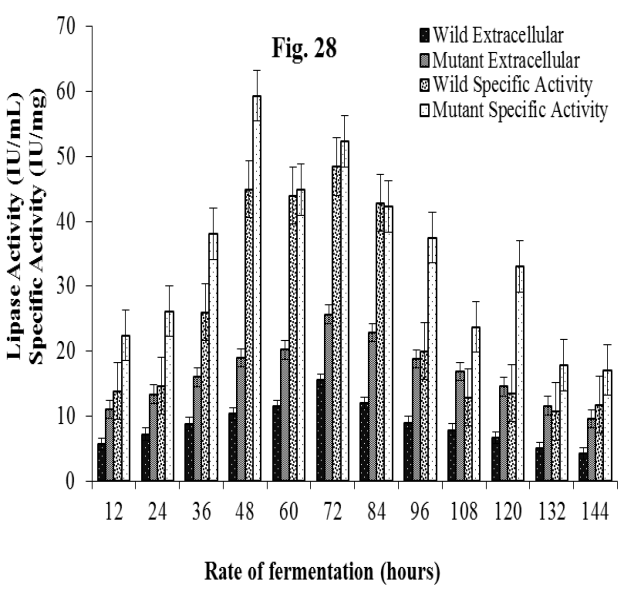
Figs. 20-25. Effect of the (fig. 20) agro-industrial byproducts (fig. 21) amount of the agro-industrial byproducts, (fig. 22) type of inoculum, (fig. 23) size of inoculum, (fig. 24) selection of the moistening agents and (fig. 25) volume of the diluent on the production of extracellular lipases & specific activity by wild (MBL-1511) & mutant (MBL-1511^{SA-4}(150 min) strains of *Aspergillus niger* through SSF. Error bars show standard deviation among three observations.











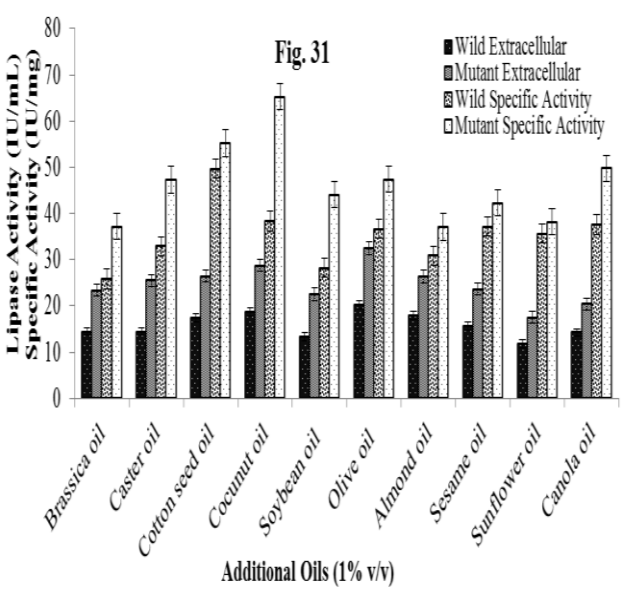


Fig. 26-31. Effect of the (fig. 26) initial pH, (fig. 27) incubation temperature, (fig. 28) rate of fermentation, (fig. 29) additional carbon sources, (fig. 30) additional organic and inorganic nitrogen sources and (fig. 31) additional oils as organic carbon sources on the production of extracellular lipases & specific activity by wild (MBL-1511) & mutant (MBL-1511^{SA-4}(150 min) strains of *Aspergillus niger* through SSF. Error bars show standard deviation among three observations.

Proximate analysis: The significant increase in protein level during fermentation may be attributed to efficient bioconversion of highly polymerized carbohydrates into microbial protein and production of enzymes which are proteinaceous in nature (Bhatnagar, 2004). It was reported that by SSF, it was possible to convert cassava to a protein enriched animal feed and the highest increase in protein content observed was 14.32% from the initial 1.28% by filamentous fungi (Mitra *et al*., 1996). Decrease in the fat content might be due to the activity of extracellular lipolytic enzyme produced by the fungus on the lipid present in the basal substrate. Our results are in conformity with previous study which indicates that fat level decreased after 48 h and which was increased after 120 h of incubation period (Joseph *et al*., 2008). This increase may be due to the utilization of easily digestible soluble carbohydrates by the growing fungus, leaving the indigestible fiber content high as reported previously (Singh *et al*., 1990). Our results support previous findings that brassica meal has high crude protein, crude fiber and high fat contents as compared to other meals before and after the fermentation which led to maximum lipases production in present studies and it may be the best source of nutrients for the fungus. Other sources may also be interchangeably used in the scale up studies. Therefore brassica meal was used in further studies.

Optimization of other cultural conditions for the production of extracellular lipases

Effect of agro-industrial by-product: Cost effective medium of enzyme production is always a cherished goal during process optimization of enzyme production (Mohseni *et al*., 2012). Brassica meal was proved a better hyper-producer among all trialed additive substrates. The reason might be due to the oil cakes of brassica meal contain significant amount of oil along with the seed proteins that become available to the lipolytic organisms as carbon and nitrogen source for their growth while the other meals didn't suitable for enzyme. Apart from this some essential nutrients and minerals are also found in meal of brassica (Dharmendra & Parihar, 2012). Apart from this some essential nutrients and minerals are also found in meals. Our results are in line with findings of other workers (Dharmendra & Parihar, 2012). Thus, brassica meal was identified as the best source of carbon and nitrogen for enhanced lipases production and used for further studies.

Effect of substrate concentration: Substrate concentration also plays a vital role for lipase production. In the present study 10g substrate gave the maximum production. The same results were reported by other researchers (Gutarra *et al*., 2005), and reason was that at high concentration of substrate, inoculum level becomes low while adequate quantity of substrates and inoculum level is required for proper growth and maximum lipases production. As the size of substrate increases, the production of lipases decreases. Therefore, 10g of substrate was optimized and used for further studies.

Effect of type of inoculum: Type of the inoculum also plays an important role for the production of extracellular lipases. Maximum extracellular lipase activity by wild was observed when 1mL of spore inoculum was used. As the size of inoculum was increased, the lipase production gradually decreased and the density of spore inoculum acts as an important variable role for the production of lipases (Gutarra *et al*., 2007). The reason might be due to the more spore's concentration present in the spore inoculum as compared to vegetative inoculum (Freire *et al*., 1997), which is in line with our results. Hence, spore inoculum was used for further studies.

Effect of size of inoculum: Size of inoculum also has great influence on the production of lipases. In the present studies maximum production was achieved by using 1mL inoculum. While current findings are not in agreement with the findings of Imandi *et al*. (2010) where 2mL of inoculum supported maximized enzyme production. The reason might be as the size of inoculum was increased, the lipase production gradually decreased. It might be due to the reason that it used up bulk of the substrate for growth and metabolic processes, hence enzyme synthesis decreased after 1mL of inoculum (Gutarra *et al*., 2007). Our results are accordance with Singh *et al*., (2006). Hence, 1mL of spore inoculum was selected for further studies.

Effect of moistening agents: Moistening agents (diluents) also play an important role in the production of lipases. In the present finding Phosphate buffer (pH 6.8) as an extractant supported highest units of lipases production. Other extractants showed the lower enzyme activity as compared to phosphate buffer (pH 6.8) reason might be due to the other extractants didn't fulfill the nutritional value. Our results are inline to (Mahanta *et al*., 2008). Therefore phosphate buffer (pH 6.8) was used for further studies.

Effect of the volume of the diluent: Volume of diluent, also plays an important role in the production of lipases. The lipase production was decreased at very high moisture content which may be ascribed to the decrease in porosity and hence decrease in gaseous exchange leading to sub optimal growth and less enzyme production as indicated by Silman *et al*. (1979). Low levels of lipases were observed with poor moisture content which resulted in the reduced solubility of nutrients of the substrate. This condition further lowers the degree of swelling and creates higher water tension as suggested by Guerra *et al*. (2003). The present results are in accordance with the results of (Mahanta *et al*., 2008). Therefore 10mL volume of the diluent was selected for further studies.

Effect of initial pH: Initial pH of the diluent also has great role for lipases production. Regarding pH of diluent, it was observed that pH 6.2 supported maximized enzyme production. The higher enzyme production was also obtained at pH of 5 (Gutarra *et al*., 2009;) and 6.5 pH by *Rhizopus chinensis* in (Sun & Xu, 2008). It was due to the reason that enzymes production is favoured in acidic

medium. It might be due to the reason that production of the enzyme by fungal source was very delicate to pH, a slight change in it might cause denaturation of the enzyme (Gombert, 1999). Our results are in line with Anbu *et al*. (2011). Therefore, pH 6 was optimized for further studies.

Effect of incubation temperature: Incubation temperature plays a dynamic role in lipases production from *A. niger* through SSF. Incubation temperature of 30ºC supported maximized lipases production both from wild and the mutant strains of *A. niger*. The fungal efficiency in enzyme construction might be due to the reason that low temperature supports the fungal growth by direct contribution of ecological temperture in metabolic synthesis (Anbu *et al*., 2011). High temperature caused inhibitory effect on growth of fungus and thus affecting the lipase productivity (Lima *et al*., 2003). Our results are accordance with (Anbu *et al*., 2011). Therefore, incubation temperature of 30°C was used for further studies.

Rate of fermentation: Rate of fermentation plays vital role in lipases production. In the present studies Incubation period after 72 h gave the maximum lipase production. Optimal lipase production was achieved at 24 h of incubation time (Mahadik *et al.,* 2004). The higher lipase productivity achieved at 96 h (Toscano *et al.,* 2011). In current study, maximum yield obtained after 72 h, it might be due to the reason that exhaustion of nutrients in substrate, which resulted in the inactivation of enzyme after 72 h of incubation period. Decline in exponential curve might be due to the exhaustion of nutrients or loss of moisture after specific incubation period. The results are in line with the findings of (Edwinoliver *et al*., 2010). Therefore, Incubation period of 72 h was used for further studies.

Effect of additional organic carbon sources; Additional carbon source also play a vital role for lipases production. In the present studies yeast extract is the best nitrogen source of lipase production. Glucose 1% gave the maximum lipases activity (Maia *et al*., 2001). It might be due to the reason it was miscible with water and did not generally inhibit fungal growth. Our finding is in line to the (Pokorny *et al*., 1994). Therefore tween 80 @ 1% (w/v) was optimized for further studies.

Effect of additional organic and inorganic Nitrogen sources: Organic nitrogen sources effect the lipase production. In the present studies yeast extract gave the maximum production. It might be due to the reason that yeast extract best source of amino acids provide the nutrients to the medium that enhanced the lipase production. Maximum units were achieved when casein was used (Elibol & Ozer, 2000). Maximum lipase units were obtained by using the organic nitrogen source of peptone (Cihangir & Sarikaya, 2004). Current finding are according to (Sangeetha *et al.,* 2009). Therefore yeast extract @ 1% (w/v) was used further.

Effect of additional oils as organic carbon sources: Organic carbon sources (various oils) produce the lipase at higher level. In the present work olive oil @ 1% gave the maximum lipase production. It might be due to that olive oil contains all essential nutrition for fungal growth Mahadik *et al*. (2002). Our findings are according to (Gutarra *et al*., 2007; Wang & Shan, 2008). Therefore olive oil @ 1% (v/v) was suggested for enhanced lipases production by wild and mutant strain of *A. niger*.

Conclusions

Isolated hyper producer strain from different environments and food items was morphologically identified. Our results suggest that *Aspergillus niger* (MBL 1511) sequence registered in NCBI database can be used as a reference for identification of fungi. Proximate analysis of agro-industrial byproducts will help in designing an industrial scale medium and identify the byproducts that can interchangeably be used for lipases production. The results will be helpful for establishment of industrial unit of lipases in order to reduce the economic burden of country.

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