Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 10 (2): 38-53, 2015
ISSN 1816-4935 / DOI: 10.3923/jm.2015.38.53

Optimization of Process Variables for Extracellular Lipase Production from *Emericella nidulans* NFCCI 3643 Isolated from Palm Oil Mill Effluent (POME) Dump Sites Using OFAT Method

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ABSTRACT

An extracellular acidic lipase producing fungi, screened and isolated from Palm Oil Mill Effluent (POME) dump sites, Pedavegi, West Godavari Dist, A.P., was found to be *Emericella nidulans* NFCCI 3643 by ribotyping of 28S rRNA gene. BLAST search following analysis of phylogenetic tree revealed that its closest phylogenetic neighbor is *Emericella fruticulosa* strain CZ032. Optimization of process variables for the enhanced lipase production from *Emericella nidulans* NFCCI 3643 was carried out using traditional OFAT (One Factor At a Time) method and found that the best production medium for highest lipase yield was Lipase Production Medium (LPM) 3 containing 1.5% olive oil as sole carbon source, 1% $(NH_4)_2SO_4$ as nitrogen source, 0.5% gum arabic as surfactant, pH 6 at 30°C and 150 rpm.

Key words: Extracellular acidic lipase, *Emericella nidulans* NFCCI 3643, POME, optimization, OFAT

INTRODUCTION

Lipases (triacyl glycerol acyl hydrolases, EC 3.1.1.3) as biocatalysts catalyzes the reversible hydrolysis of fats and oils (Gilbert, 1993). They are ubiquitous and are widely distributed in the nature in all sources like plants, animals, fungi, bacteria, archae and yeast (Saxena *et al*., 2003; Salihu *et al*., 2012). However, microbial lipases are preferred choices because of their high yield, low production cost, ease of genetic manipulation, broad substrate specificity and stability in organic solvents (Shu *et al*., 2010). Lipases play a variety of roles in the biological systems like esterification, resolution of chiral substrates and trans esterification etc. (Padilha *et al*., 2012). The main advantages of using enzymes for hydrolytic reactions rather than chemicals involves requirement of less energy and higher quality of the obtained products. Lipases catalyze reactions in both aqueous and non aqueous media because of their ability to use different substrates, organic solvents and their ability to survive at broad temperatures and pH ranges (Saxena *et al*., 2003).

Among micro organisms, fungi are preferred microbes as they thrive well in extreme environmental habitats because of the presence of efficient enzyme systems and are considered to be cheapest sources of lipase production (Iftikhar *et al*., 2012). Among fungi, especially *Aspergillus* spp., *Mucor* spp., *Rhizopus* spp. and *Penicillium* spp., are preferable lipase sources (Mahadik *et al*., 2002; Rekha *et al*., 2012).

Fermentation medium optimization plays critical role in enhancing the production yields and is important for industrial application. Optimization of process variables is usually done by sub merged fermentation process and it is the best choice among various other fermentation techniques (Li and Zong, 2010). Optimization for enhanced enzyme production not only depends upon medium components like carbon, nitrogen sources and surfactants but also on physical parameters like pH, temperature and agitation. In general carbon, nitrogen sources and surfactants play important role as media components and were also reported to be main factors for enhancing lipase production by Guerzoni *et al*. (2001). There were also reports regarding the enhanced lipase production in the presence of lipidic substrates (oils), various triacyl glycerols, fatty acids etc (Gupta *et al*., 2004).

The objective of the present study was to optimize process variables for enhancing lipase production by *E. nidulans* NFCCI 3643 isolated from POME dump sites.

MATERIALS AND METHODS

Microorganism and culture conditions: The fungal culture used in the present study, *Emericella nidulans* was screened and isolated from Palm Oil Mill Effluent (POME) dump sites, Pedavegi, West Godavari District. Culture was maintained on the 4% Potato Dextrose Agar (PDA) slants throughout the study period. Based on morphological (plate morphology observation) and microscopic observations (Lacto phenol cotton blue staining) the culture was identified as *Emericella nidulans*. The culture was deposited at National Fungal Culture Collection of India (NFCCI), Agarkhar Research Institute, Pune for further molecular identification.

Molecular identification of the culture: For molecular identification, the fungal genomic DNA was isolated in pure form *Emericella nidulans*. The D1 and D2 region (LSU) of rDNA was successfully amplified using fungal universal primers (LROR and LR7). The PCR was set up with ABI-BigDye® Terminatorv3.1 Cycle sequencing kit. The raw sequence obtained from ABI 3100 automated DNA sequencer was manually edited for inconsistency. The sequence data was aligned with publicly available sequences using BLAST and analyzed to reach identity of the organism. The evolutionary relationship of the organism was also determined by the construction of a phylogenetic tree using phylogeny.fr (Dereeper *et al*., 2008).

Optimization of process parameters by OFAT (One factor at a time) method: For the optimization of process variables using OFAT method, Lipase Production Medium 3 (LPM 3) was used to optimize various fermentation parameters like temperature, pH, agitation, inoculum concentration, substrates, additional carbon sources, nitrogen sources and surfactants using Submerged Fermentation (SmF). The LPM 3 consists of (g L^{-1}), KNO_3 (2.5), KH_2PO_4 (1), $MgSO_4$ (0.5) and olive oil (15). The pH of the medium was adjusted to 5.6 using citrate phosphate buffer. Cultivation was carried out in 250 mL Erlenmeyer flasks containing 45 mL medium and inoculated with 3% inoculums and incubated for 4 days in an orbital shaker (150 rpm) at 30°C. Fungal biomass was separated by centrifugation at 10,000 rpm for 10 min and the filtrate was considered as a crude enzymatic extract and stored at -20°C for further use.

Biomass determination: The fungal mycelia obtained by filtering the culture broth through Whatman filter paper No. 1 was dried at 80°C until a constant weight is attained (Silva *et al*., 2005). Dry weight of the fungal biomass was calculated and expressed as mg mL^{-1}. Values were the mean of three sets of experiments run simultaneously.

Extracellular lipase assay: Yield of p-nitrophenol was used to measure lipase activity with p-nitrophenylpalmitate (pNPP) (Sigma, USA) as the substrate (Maia *et al*., 2001). The assay mixture consisted of 100 µL of sample and 900 µL of substrate solution containing 10 mg of pNPP dissolved in 1 mL of propan-2-ol diluted in 9 mL of 50 mM Tris-HCl pH 7.0 containing 40 mg of Triton X-100 and 10 mg of gum arabic. The assay mixture was incubated at 30°C for 30 min and the p-nitrophenol released was measured at 410 nm.

One unit of activity was defined as the amount of enzyme that liberated 1 µmol of p-nitrophenol per min under the assay conditions.

Lipase Production Media (LPM): Initially Lipase production by *Emericella nidulans* NFCCI 3643 was studied using various production media reported earlier. Table 1 shows the composition of 5 different Lipase Production Media (LPM) that were used for culture media optimization.

RESULTS

Identification of the fungal culture: The fungal culture was identified as *Emericella nidulans* NFCCI 3643 based on morphological (Fig. 1), microscopic (Fig. 2) and molecular identification. The fungal culture was deposited at NFCCI with an accession number 3643. Figure 3 shows the D1 and

Table 1: Composition of 5 different Lipase Production Media (LPM) that were used for culture media optimization

LPM 1 (g L^{-1})	LPM 2 (g L^{-1})	LPM 3 (g L^{-1})	LPM 4 (g L^{-1})	LPM 5 (g L^{-1})
Peptone-20	Glucose-10	KNO_3-2.5	Glucose-10	$MgSO_4.7H_2O$-0.5
Yeast extract-5	Peptone-20	KH_2PO_4-1	$(NH_4)_2S0_4$-5	KCL-0.5
NaCl-5	NaCl-5	$MgSO_4$-0.5	Na_2HPO_4-6	Yeast extract-0.25
Na_2C0_3-0.25	Yeast extract-5	Olive oil-15 mL	KH_2PO_4-2	Peptone-0.25
Olive oil-10 mL	$(NH_4)_2S0_4$-5	pH-5.6	$MgSO_4$-3	Glucose-12.5
pH-8	Na_2HPO_4-6		$CaCl_2$-3	Olive oil-12 mL
	KH_2PO_4-2.0		Olive oil-10	pH-7
	$MgSO_4$-3		pH-5	
	$CaCl_2$-3			
	pH-6			

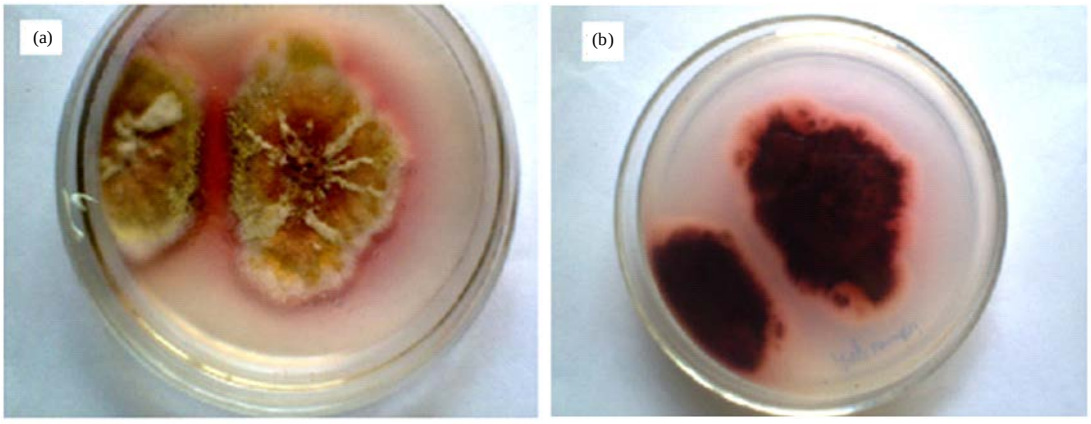

Fig. 1(a-b): Colony morphology of *Emericella nidulans* NFCCI 3643 on Czepakdox Agar Plates (CZA), (a) CZA plate showing green colonies which slowly turn to brown color ascospores and (b) Reverse of CZA plate where colonies appear as orange red to purple red

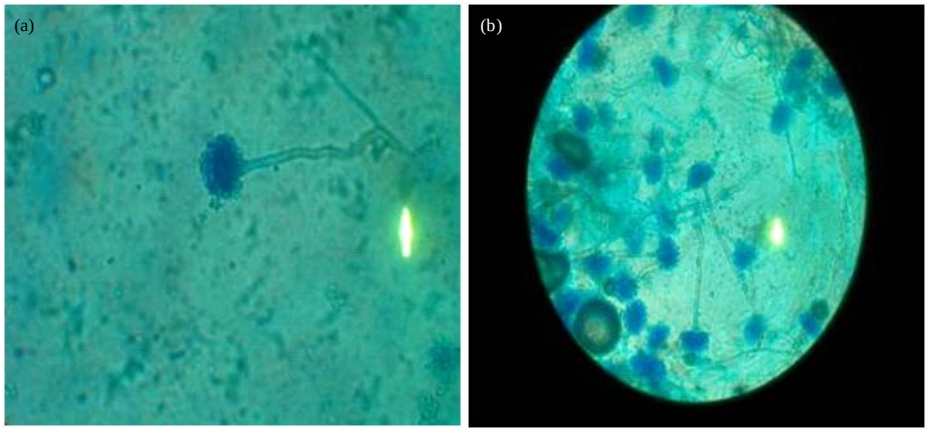

Fig. 2(a-b): Microscopic images of *Emericella nidulans* NFCCI 3643 obtained by lacto phenol cotton blue staining of fungal spores

GCCCGTGCCCGTGTGAAGANCCTTCGACGAGTCGAGTTGT
TTGGGAATGCAGCTCTAAATGGGTGGTAAATTTCATCTAA
AGCTAAATACCGGCCGGAGACCGATAGCGCACAAGTAGA
GTGATCGAAAGATGAAAAGCACTTTGAAAAGAGAGTTAA
ACAGCACGTGAAATTGTTGAAAGGGAAGCGCTTGCGACC
AGACTCGGCCCCGGGGTTCAGCCAGCACTCGTGCTGGTGT
ACTTCCCCGGGGGCGGGCCAGCGTCGGTTTGGGCGGCCGG
TCAAAGGCCCCAGGAATGTATCGCCCTCCGGGGTTGTCTT
ATAGCCTGGGGTGCAATGCGGCCAGCCCGGACCGAGGAA
CGCGCTTCGGCACGGACGCTGGCGTAATGGTCGCAAACGA
CCCGTCTTGAAACACGGACCAAGGAGTCTAACATCTACGC
GAGTGTTCGGGTGTCAAACCCGTACGCGCAGTGAAAGCGA
ACGGAGGTGGGAGCCCCCCCGGGGCGCACCATCGACCGA
TCCTGATGTCTTCGGATGGATTTGAGTAGA

Fig. 3: 28S rRNA gene sequence of *Emericella nidulans* NFCCI 3643. The D1 and D2 region (LSU) of r DNA sequence of *E. nidulans* NFCCI 3643 obtained by PCR amplification of genomic DNA isolated from *E. nidulans* NFCCI 3643 using fungal universal primers LROR and LR7

D2 region (LSU) of rDNA sequence amplified from *E. nidulans*. From the phylogram (Fig. 4), it is inferred that *E. nidulans* NFCCI 3643 has close similarity to *Emericella fruticulosa* strain CZ032.

Selection of Lipase Production Medium (LPM): Of the five different lipase production media that were tested, it was observed that LPM 3 is able to produce highest lipase activity (Fig. 5). Hence, LPM 3 was selected for further optimization studies.

Effect of incubation period: The lipase production as factor of incubation time was studied with LPM 3 in shake flasks up to 144 h. From the data represented in Fig. 6, it was evident that maximum yield was obtained after 96 h of incubation period.

Effect of substrates: The suitable substrate for optimum lipase production was analyzed by adding various oils as substrates to LPM 3 viz., Olive oil, Sunflower oil, Ground nut oil, Palm oil,

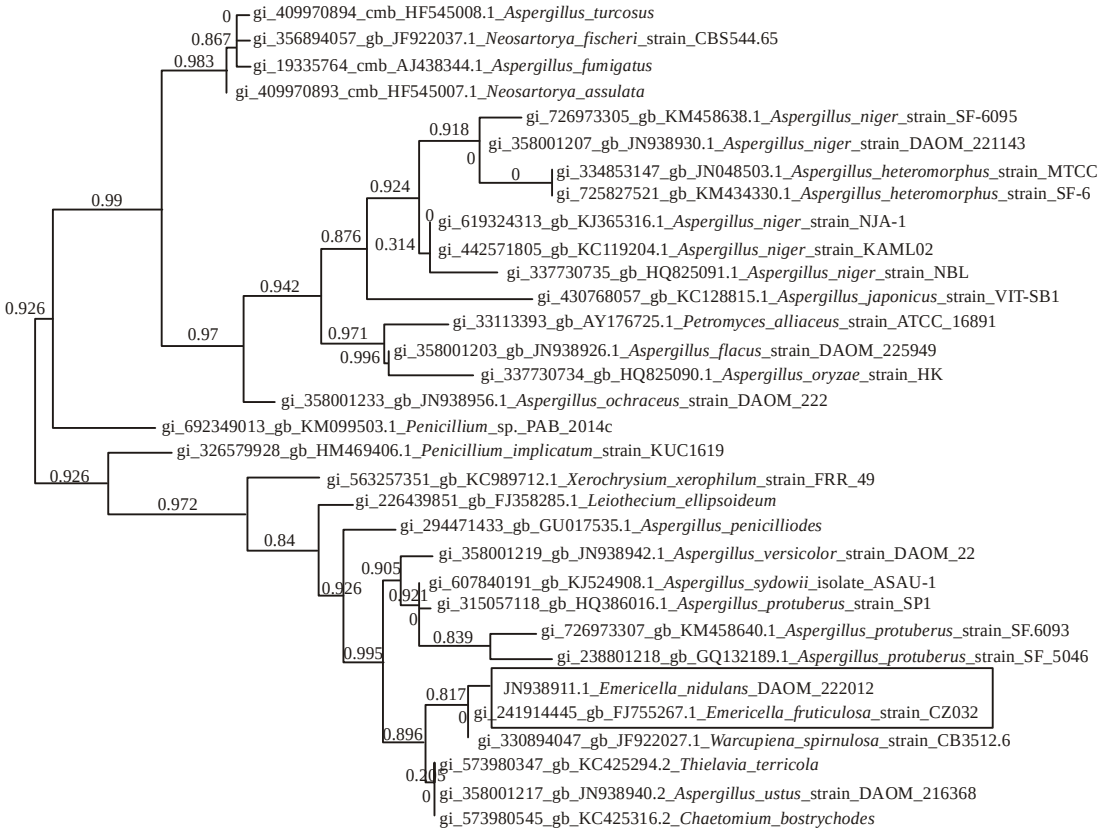

Fig. 4: Phylogenetic tree of *E. nidulans* NFCCI 3643 constructed by aligning the rDNA sequence of *E. nidulans* with publicly available sequences using BLAST following tree construction using phylogeny.fr

Gingelly oil, Coconut oil and Cotton oil. From the results presented in Fig. 7, it was evident that olive oil is the best substrate for lipase induction by *Emericella nidulans*.

Effect of temperature: The effect of temperature on enzyme production was studied by incubating the fungus at different temperatures from 10-60°C and it was found that the enzyme production was maximum at 30°C (Fig. 8).

Effect of the medium pH: The effect of pH on lipase production was studied by culturing the fungus in LPM 3 for 96 h with a pH range 4-10 and it was found that optimum pH for lipase production was pH 6. However, the organism is able to show optimum lipase activity over a broad range of pH (Fig. 9).

Effect of agitation: Agitation increases the metabolism as the organism is capable of utilizing oxygen very well. In the present study, effect of agitation on lipase production was studied by incubating the organism in a rotary shaker incubator at 0 (stationary) 100, 150 and 200 rpm. The results (Fig. 10), obtained shows that lipase production was maximum at 150 rpm.

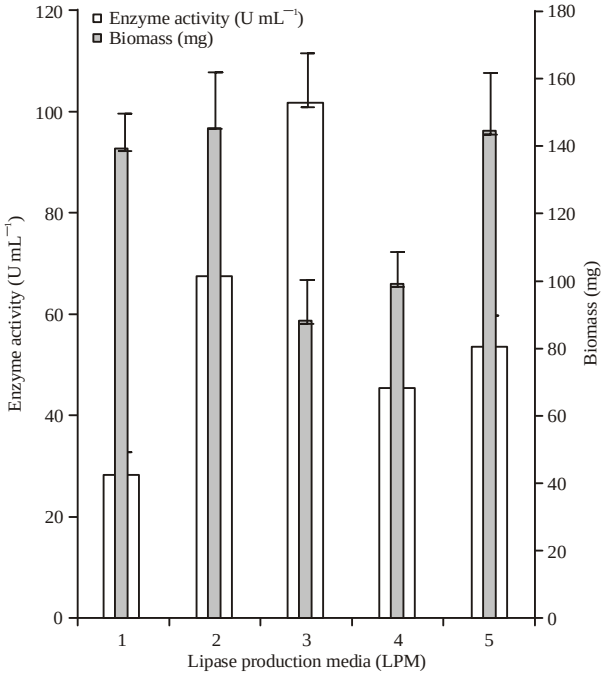

Fig. 5: Effect of different lipase production media on lipase production-spore suspension of *E. nidulans* NFCCI 3643 was inoculated into different lipase production media viz., LPM 1, 2, 3, 4 and 5 are incubated at 30°C for 4 days at 150 rpm and enzyme activity was estimated

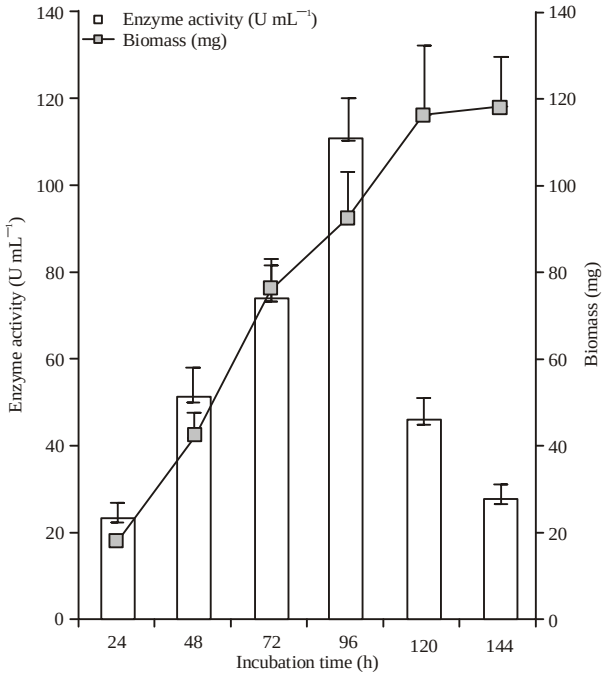

Fig. 6: Effect of incubation time on lipase production-spore suspension was inoculated into LPM 3 and incubated for different time periods viz., 24, 48, 72, 96, 120 and 144 at 30°C followed by enzyme estimation

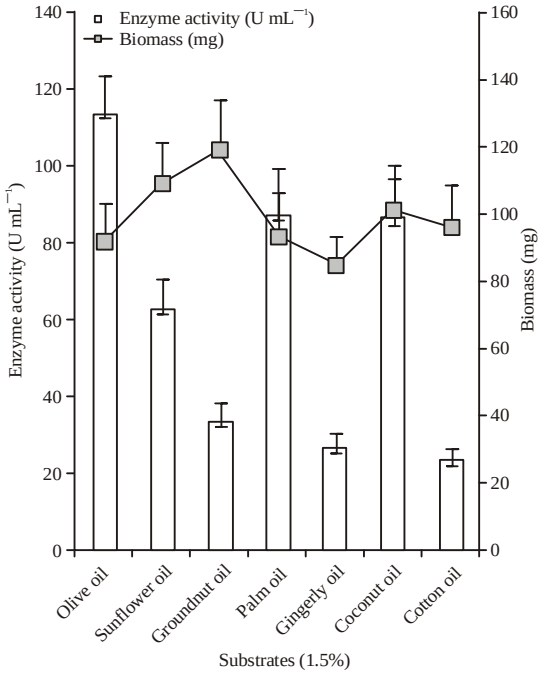

Fig. 7: Effect of different oil substrates on lipase production-spore suspension was inoculated into LPM 3 with 1.5% each of various substrates viz., olive oil, sunflower oil, ground nut oil, palm oil, gingelly oil, coconut oil and cotton oil and incubated at 30°C for 96 h at 150 rpm and enzyme activity was estimated

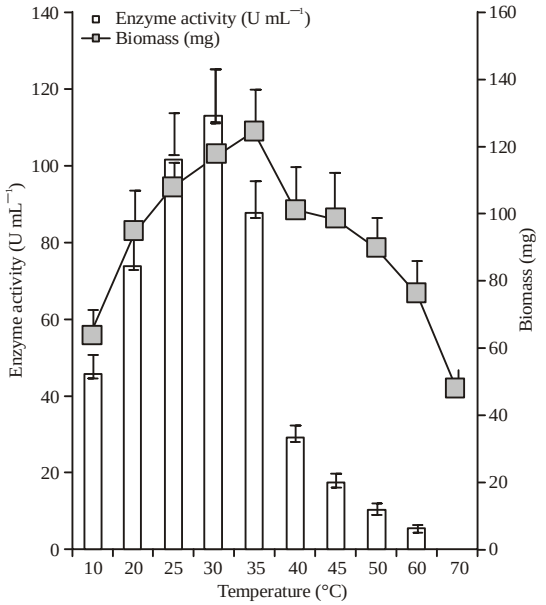

Fig. 8: Effect of incubation temperature on lipase production-spore suspension was inoculated into LPM 3 containing olive oil as substrate and incubated at different temperatures from 10-70°C for 96 h at 150 rpm and enzyme activity was estimated

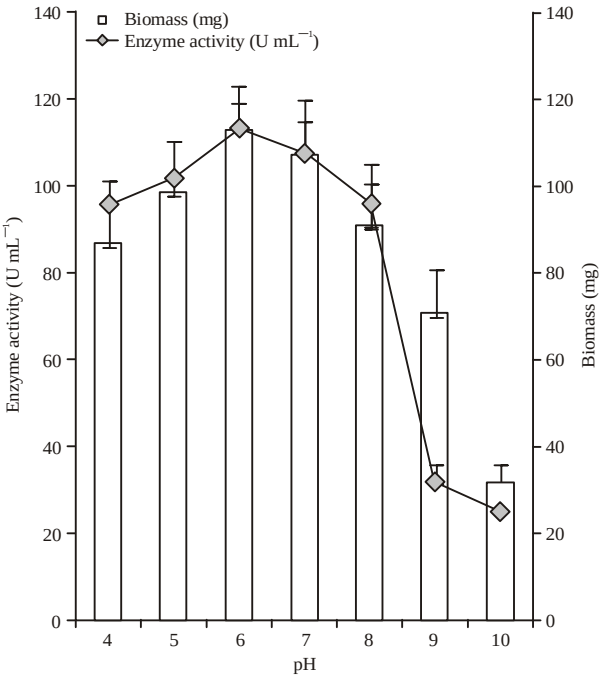

Fig. 9: Optimization of pH for lipase production-spore suspension was inoculated into LPM 3 containing olive oil as substrate with varying pH ranging from 2-10 and incubated at 30°C for 96 h at 150 rpm and enzyme activity was estimated

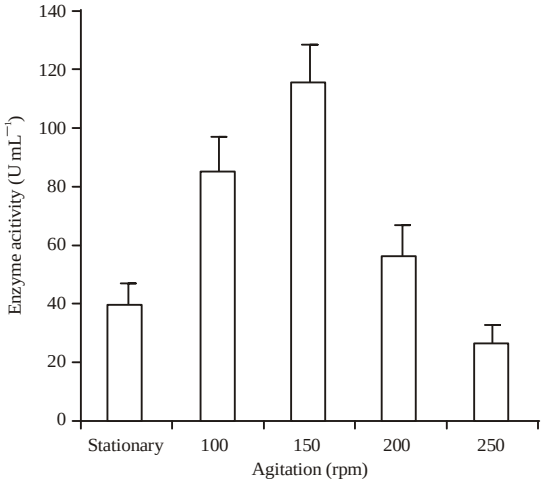

Fig. 10: Effect of agitation on lipase production-spore suspension was inoculated into LPM 3 of pH 6 containing olive oil as substrate incubated both under static (0) and shake flask conditions (100, 150, 200 and 250 rpm) at 30°C for 96 h and enzyme activity was estimated

Effect of inoculum concentration: The effect of inoculum concentration on lipase production was studied by incubating the organism in a production medium containing different concentrations (1-10%) of the inoculum and from the results (Fig. 11) it was found that a variation in inoculum concentration does not affect the enzyme production much.

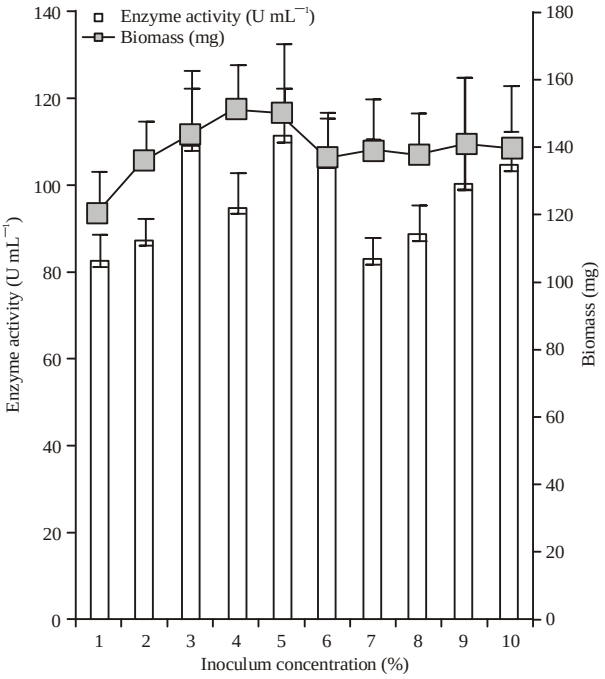

Fig. 11: Effect of inoculum concentration on lipase production-spore suspension containing different concentrations of inoculum (1-10%) was inoculated into LPM 3 of pH 6, containing olive oil as substrate and incubated at 30°C for 96 h and enzyme activity was estimated

Effect of additional carbon sources: To check whether carbon source in the form of carbohydrates affect the lipase production, various sugars viz., Glucose, Fructose, Galactose, Arabinose, Maltose and Sucrose at a concentration of 1% were added to LPM containing olive oil as primary substrate. The data obtained (Fig. 12) indicates that no sugar added as additional carbon sources enhanced the enzyme production compared to control.

Effect of organic and inorganic nitrogen sources: Effect of organic and inorganic nitrogen sources on the lipase production was studied by replacing the nitrogen source of LPM 3 with various organic (1%) and inorganic nitrogen sources (1%) such as Peptone, Yeast extract, Malt extract, Beef extract, Ammonium sulphate, Ammonium nitrate, Ammonium dihydrogen phosphate, Ammonium chloride, Sodium nitrate etc., with olive oil as substrate. Though the enzyme production was also significantly enhanced with yeast extract, an organic nitrogen source but the production was maximum with ammonium sulphate, an inorganic nitrogen source (Fig. 13a-b).

Effect of surfactants: Effect of various surfactants like SDS, PEG, Tween 20, Tween 80, Gum arabic and triton X100 on the lipase production was studied by adding them at a concentration of 0.5% to LPM 3 containing olive oil as substrate, ammonium sulphate as nitrogen source with pH 6 and the culture flasks were incubated at 30°C for 96 h at 150 rpm. The data from Fig. 14 shows that lipase production was maximum with gum arabic as surfactant compared to control without surfactant.

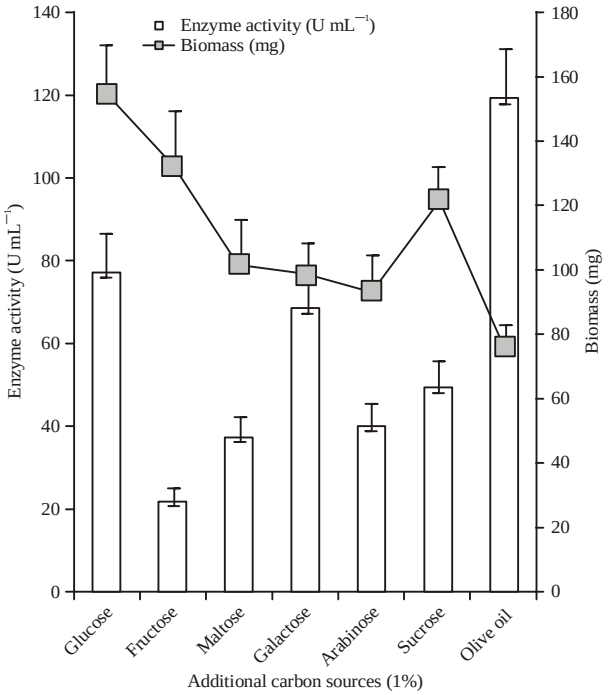

Fig. 12: Effect of different sugars as additional carbon source on lipase production-Spore suspension was inoculated into LPM 3 of pH 6 containing olive oil and 1% each of various additional carbon sources Viz., Glucose, Fructose, Galactose, Arabinose, Maltose and Sucrose and incubated at 30°C for 96 h and enzyme activity was estimated

DISCUSSION

Lipases have the ability to mediate reactions in organic solvents as they possess low water activity which drives the reacting molecules towards synthesis rather than hydrolysis (Gumel *et al*., 2011). It was known facts that oil enriched areas are potential sites for the growth of lipase producing micro organisms. Palm Oil Mill Effluent (POME) dump sites are usually enriched with oil so can be used for screening and isolation of potential lipolytic microorganisms.

A group of lipase producing organisms were screened and isolated from the soil samples collected from POME dump sites, Pedavegi, West Godavari district. A hyper producer of extracellular lipase was selected and submitted for molecular identification at NFCCI, Agarkhar Research Institute, Pune and confirmed that the fungus was *Emericella nidulans* strain NFCCI 3643 and the strain was also deposited at NFCCI with an accession number 3643. Phylogenetic analysis revealed that the fungus showed close similarity with *Emericella fruticulosa* CZ032.

Optimization of process variables offers enhanced enzyme yield. Submerged fermentation is the most efficient method among various other fermentation techniques. In general the extracellular lipase production is influenced by various physical and chemical parameters like, the culture pH, the growth temperature, type and concentration of carbon and nitrogen sources, presence of surfactants, agitation and inoculums concentration.

Of the different lipase production media that were examined for optimal lipase production, LPM 3 showed highest activity. The sole carbon source in this medium is olive oil alone and the fungus had to break down olive oil for its growth and metabolism. The fat degradation was

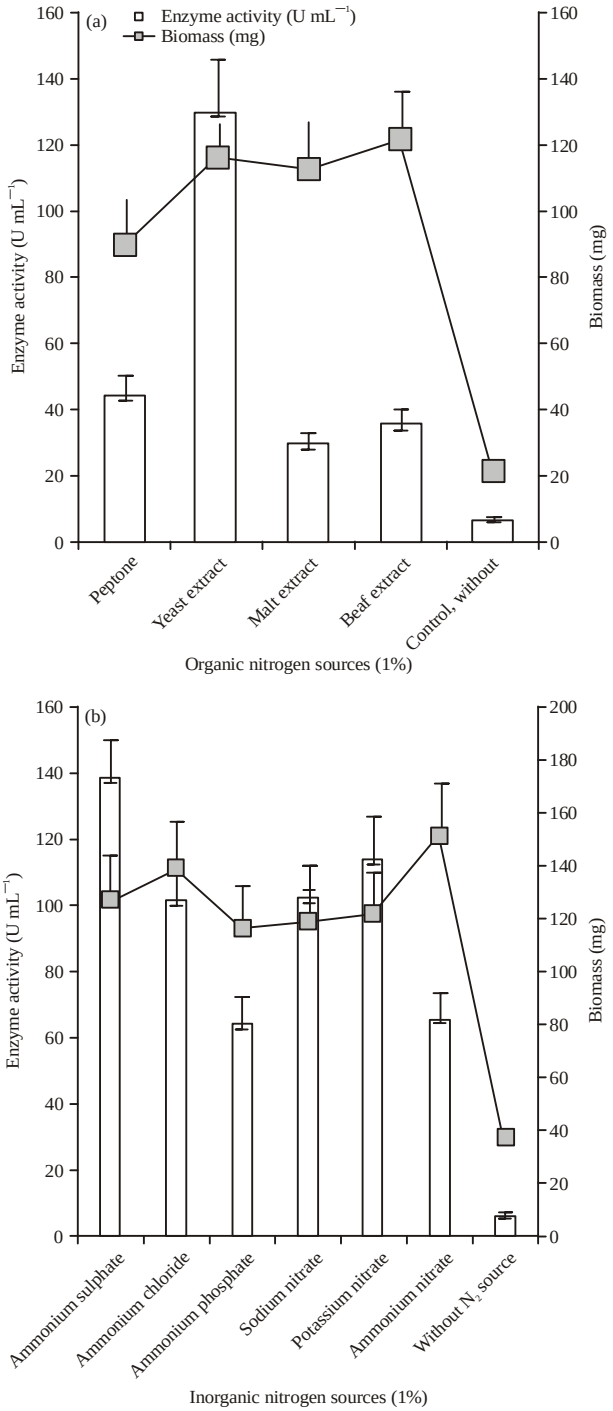

Fig. 13(a-b): Effect of organic and inorganic nitrogen sources on lipase production-spore suspension was inoculated into LPM 3 of pH 6 containing olive oil as substrate with 1% each of various organic and inorganic nitrogen sources viz., peptone, yeast extract, malt extract, beef extract, ammonium sulphate, ammonium nitrate, ammonium di hydrogen phosphate, ammonium chloride, sodium nitrate and incubated at 30°C for 96 h and enzyme activity was estimated

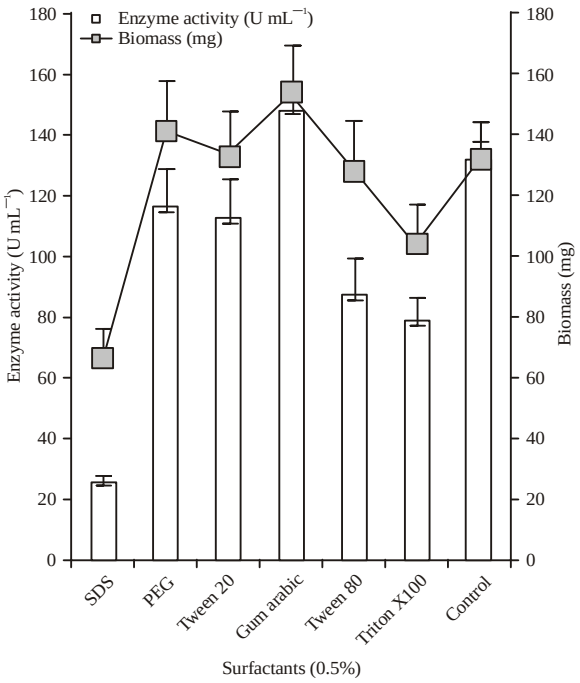

Fig. 14: Effect of surfactants on lipase production-spore suspension was inoculated into LPM 3 of pH 6, olive oil as substrate and ammonium nitrate as nitrogen source with 0.5% each of various surfactants viz., SDS, PEG, Tween 20, Tween 80, Gum arabic and Triton X100 and incubated at 30°C for 96 h and enzyme activity was estimated

mediated by lipases. This could be the main reason that the fungus showed highest enzymatic activity in LPM 3 though the biomass produced in LPM 3 was less compared others.

Incubation time also plays an important role in lipase biosynthesis. In the present study, an incubation period of 96 h under submerged fermentation was found to be optimum for enhanced lipase production by *Emericella nidulans* NFCCI 3643. Different fungi require different incubation periods for optimum lipase production. The similar incubation time, i.e., 96 h was reported for maximum lipase activity in *A. terreus* by Gulati *et al*. (1999), in *Rhizopus arrhizus* by Yang *et al*. (2005), in *Fusarium solani* FS1 (Maia *et al*., 2001). An incubation time of 72 h was reported for maximal lipase production in *A. niger* MTCC 2594 (Edwinoliver *et al*., 2010), in *R. chinensis* (Sun and Xu, 2008), in *Penicillium chrysogenum* (Kumar *et al.,* 2011).

Most of the microbial lipases are inducible and secrete extracellular enzymes in to the surrounding environment upon induction. These inducible extracellular enzymes in general are produced in the presence of inductors, such as fatty acids, hydrolysable esters or of a lipid such as oil or triacylglycerol or Tween, bile salts and glycerol. In the present study on optimization of process variables, lipase production was enhanced when olive oil was used as substrate. Lipase production increases with relative increase of C18: n fatty acid esters in the respective vegetable oil (Lakshmi *et al*., 1999). Olive oil is considered as the best inducer of lipase production among different vegetable oils (Papanikolaou *et al*., 2011; Nunes *et al*., 2011).

Temperature is also one of the critical parameter that needs to be optimized and it varies from organism to organism. Increasing the temperature fastens the physiological processes up to certain

level beyond which there will be a reversible effect. Extracellular lipase production and secretion are also greatly influenced by the temperature as it enhances the production by changing the physical properties of the cell membrane. The optimum temperature for lipase production by *Emericella nidulans* was found to be 30°C and there was a gradual decrease in enzyme production and activity beyond 30°C. This observation is in agreement with the temperature optima of 30°C reported for lipase production in *Penicillium verrucosum* by Pinheiro *et al*. (2008).

Many of the biological processes require optimum physiological pH and this is absolute for enzymes as they show highest activity at their optimum pH. The initial pH of the fermentation medium influences the rate of extracellular lipase production. Optimization of lipase production by *Emericella nidulans* NFCCI 3643 over varied range of pH, revealed that pH 6 was optimum, indicating that the lipase is acidic lipase, however the enzyme production was relatively stable between the pH ranges 3-8 indicating that the organism is having broad pH specificity in terms of lipase production. Most of the lipases belonging to the *Aspergillus* spp have their pH optima in the range of 6.0-7.5, like the lipases from *A. japonicas* (Jayaprakash and Ebenezer, 2012), *A. awamori* (Romero *et al*., 2012) etc.

Agitation also had an effect in the enzyme production. From the results obtained, it is inferred that agitation is required for lipase production. This was in consistent with the results obtained by Cihangir and Sarakaya (2004). Enzyme production even occurred under stationary conditions (>35%) but increased agitation increased enzyme production and the production was optimum at 150 rpm. Increased aeration thereby increased oxygen transfer rate thereby better growth, increased dispersability of lipidic substrate under agitated conditions could be the main reasons for enhanced production of the enzyme. However, a decrease in the enzyme production at higher agitation rates was found which may be due to fragmentation of the mycelium because of shearing stress on the organism (Gulati *et al*., 2000). In the present study it was very much clear that variation in the level of inoculum size from 1-10% did not show much effect in the rate of enzyme production.

Several of the studies revealed that lipase production was significantly enhanced when lipidic substrates were used as sole carbon sources rather than with the combination of lipidic substrates and simple sugars (Zhang *et al*., 2009). In the present optimization study using OFAT method, none of the carbon sources added as additional carbon sources had effect in enhancing enzyme production. All the sugars that were added to the medium along with olive oil significantly decreased enzyme production compared to control where olive oil was the sole carbon source. Similar findings were reported by Mayordomo *et al*. (2000). This effect could be probably due to repression of the synthesis of the enzyme in the presence of simple carbon sources as the microbes prefers simple carbon sources rather than complex sources for getting energy. This result is in contrast to the results obtained by Akhtar *et al*. (1980) in case of *Mucor hiemalis* and *Aspergillus wentii*, where presence of glucose as additional carbon source enhanced lipase production.

In addition to carbon sources, nitrogen sources are also required for growth and enzyme production. In the present study, among different organic nitrogen sources tested, yeast extract showed enhanced enzyme production compared to peptone, malt extract and beef extract which significantly lowered enzyme production. Among various inorganic nitrogen source that were tested ammonium sulphate enhanced lipase production. When compared to yeast extract ammonium sulphate was found to be best nitrogen source.

Presence of surfactants also enhances extracellular lipase production by microorganisms. This could be due to the fact that surfactants can increase the cell permeability and there by facilitates

the export of several molecules across the cell membrane, including protein secretion. In addition surfactants also facilitate the contact between enzyme and substrate. The effect of surfactants in enhancing lipase production was studied in several microorganisms with varied results like presence of Triton X-100 enhances lipase production by 2 fold in *Aspergillus niger* (Mahadik *et al*., 2002) and SDS and Tween 80 were found to be best inducers in *Metarhizium anisopliae* (Silva *et al*., 2005). In the present study, effect of various surfactants like SDS, PEG, Tween 20, Gum arabic, Tween 80, Triton X-100 on lipase production was studied and found that addition of Gum arabic to the fermentation medium enhanced lipase production compared to control whereas addition of SDS has an inhibitory effect on enzyme production. SDS was also found to inhibit enzyme production in case of *Aspergillus caneus* (Saxena *et al*., 2003). Presence of surfactants in the fermentation medium will not always enhance lipase production and their effects on enzyme yields depends on both surfactant added and the strain used for the study (Dominguez *et al*., 2003).

CONCLUSION

In the present study, optimization of process variables for enhanced lipase production by *Emericella nidulans* NFCCI 3643, screened and isolated from POME dump sites was carried out by using traditional OFAT method and the best process parameters were found to be olive oil as best and sole carbon source, $(NH_4)_2S0_4$ as nitrogen source, gum arabic as surfactant, pH 6, temperature 30°C and agitation 150 rpm.

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