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Lipase Production using *Aspergillus japonicus* MF-1 through Biotransformation of Agro-Waste and Medicinal Oil Effluent

Nathan Vinod Kumar[1,2]*, Mary Esther Rani[1], R. Rathinasamy Gunaseeli[3] and Narayanan Dhiraviam Kannan[4]

[1]Research Centre, Department of Botany and Microbiology, Lady Doak College, Madurai - 625002, Tamil Nadu, India
[2]National Institute of Oceanography-CSIR, Regional Centre, Cochin 682018, Kerala, India
[3]Center for Environmental Studies, Lady Doak College, Madurai -625002, India
[4]Department Plant Biotechnology, School of Biotechnology, Madurai Kamaraj University, Madurai-62502i, Tamil Nadu, India
**Corresponding author*

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A B S T R A C T

Oil rich effluents are always a menance for many industries in the process of effluent treatment. The process usually requires more complex methods to resolve the issue to reach to disposable effluent standards. Enzymatic degradation was proved to be promising for the efficient treatment of oil rich effluents. Among the hydrolytic enzymes, lipases are the second most widely-used enzymes in industrial applications. A potential lipolytic fungus obtained from a marine soil sample exposed to oil spillage was identified as *Aspergillus japonicus* MF-1. A maximum activity of 266 U/g was observed using solid state fermentation (SSF) utilizing groundnut oil cake. Lipase activity was found to be enhanced by oxidizing and reducing agents. Medicinal oil effluent was biotransformed to produce lipase under submerged fermentation to achieve a maximum activity of 153.4 U/ml. For lipase production using *A. japonicus* MF-1, solid state fermentation was found to be a cost effective and better strategy. Moreover, the usage of medicinal oil like effluents for lipase production is quite promising for bio-remediation of oil rich effluents.

Introduction

Lipases (EC 3.1.1.3) the most versatile enzyme class are enzyme that hydrolyzes triacyl glycerides liberating fatty acids. They possess unique characteristics like substrate specificity, region-specificity and chiral selectivity (Castro-Ochoa *et al.*, 2005). The use of this enzyme has enormous potential to reduce energy requirements and solve many environmental problems especially related to industrial effluents (Pereira *et al.*, 2013). The lipase enzyme is widely used in many industries including textile, food, pulp and paper, fat and oleo chemical, pharmaceutical and more recently in biofuel industries (Singh and Mukhopadhyay, 2012). Lipases could be used for removal of hydrophobic components of wood consisting mainly of triglycerides and waxes, which cause severe problems in paper manufacturing process. There are many fungi utilized for the application of lipase production. Among them, *Aspergillus*, *Penicillium* and *Rhizopus* are the most

common and potential genera for lipase production. *Aspergillus sp.* is widely used for the production of hydrolytic enzymes such as amylase and lipase on commercial scale (Perrone *et al.,* 2008). *A. japonicus* isolated form olive oil mill waste exhibited lipolytic as well as cellulolytic activity (Gopinath *et al.,* 2005).

Submerged fermentation has its own advantages in the consideration of scaling up in industrial level. There are reports on utilization of oil mill and winery waste for the lipase production (Salgado *et al.,* 2014). However the factors to achieve the maximum enzyme activity may vary from each species and there is a need for optimization of the production medium. Filamentous fungi have the ability to grow on solid substrates and produce many extracellular enzymes (Vishwanatha *et al.,* 2010). Due to the lower production cost, solid state fermentation which usually utilizes the agro-waste as substrates is the best strategy for enzyme production on a commercial scale. Gombert *et al.,* (1999) reported the use of babassu oil cakes for lipase production using *Penicillium restrictum*. Other agro-waste utilized for lipase production includes brans (wheat, rice, soyabean and barley), oil cakes (soy, olive and gingelly) and sugarcane bagasse (Godoy *et al.,* 2011; Salihu *et al.,* 2012).

The present work focuses on achieving maximum lipase activity from *A. japonicus* under submerged and solid state fermentation (SSF) and to make a comparison of submerged and SSF. The oil cakes and effluents rich in oil were also utilized for lipase production using the fungal isolate. A high lipase activity was achieved using submerged as well as under SSF by *A. japonicus* MF-1 isolate. Production optimization and kinetics were studied in detail using suitable statistical models.

Materials and Methods

Isolation and screening of lipolytic fungi

Oil contaminated soil samples from mangrove and coastal environments of Kerala and Tamil Nadu were collected aseptically. Serial dilution was performed up to 10^{-6} dilution to isolate the distinct fungal colonies on Potato Dextrose Agar (PDA) plates at 28±2°C after 3-5 days of incubation. Isolated fungal colonies were further stored on PDA slants for screening. Primary screening was performed on PDA plates fortified with 2% olive oil as substrate and supplemented with 0.5% phenol red as pH indicator. Lipolytic fungal strains converted substrate into simpler fatty acids and changed the pH of the medium from pink to yellow (Singh *et al.,* 2006). Change in plate colour was considered positive for lipolytic activity. Secondary screening was performed by quantification of lipase activity using standard method described in the following section.

Molecular characterization of fungal isolate

DNA was isolated from the fungal isolate by using the method reported earlier (Melo *et al.,* 2006). Quality of the DNA was evaluated by spectrometric analysis as well as by performing electrophoresis on 0.8% agarose gel. DNA was further amplified using DR [5'-GGTCCGTGTTTCAAGACGG-3'] and DF [5'-ACCCGCTGAACTTAAGC-3'] universal primers for amplification of LSU 28S rDNA (Kurtzman and Robnett, 1997). The PCR reaction was performed using the method described by Vinod *et al.,* (2014). Resultant PCR amplicon was purified and sequenced using automated DNA sequencing on ABI 3730xl DNA analyzer (Applied Biosystems, USA). The sequencing chromatogram was analyzed to extract the sequence and used for BLASTn analysis against non-redundant

NCBI database which resulted in the identification of ten similar sequences. Clustal W multiple sequence alignment (Thompson *et al.,* 1994) was performed using BioEdit 5.0 and phylogenetic tree was constructed for the aligned sequences in MEGA 5.0 (Tamura *et al.,* 2011) based on neighbour joining method (Saitou and Nei, 1987).

Lipase assay

Lipase assay was performed using standard method which is described as follows. 2.5 ml of water was added into test and blank test tubes followed by 1ml of 100 mM Tris HCl buffer [pH 7.2]. 3 ml of olive oil was added as the substrate and mixed well and incubated for 5 min. 1ml of enzyme was added into test sample and incubated for 30 min at room temperature. After the incubation, 1 ml of 95% ethanol was added to stop the reaction and titrated against 0.1 M NaOH with 0.9% phenolphthalein as an indicator. Appearance of pale pink colour was considered as end point. One unit of lipase is defined as the amount needed to hydrolyze 1.0 microequivalent of fatty acid from a triglyceride in one hour at pH 7.7 at 37°C.

Utilization of ayurvedic oil effluent for lipase production

Ayurvedic pharmaceutical effluent rich in oil and higher forms of hydrocarbon were obtained from commercial ayurvedic drug manufacturer's in Madurai, Tamil Nadu. It was made into different concentrations of 25, 50 and 100% (v/v) using phosphate buffer [pH 8.2]. *A. japonicus* MF-1 was inoculated into the sterilized effluent which was used as sole production media component and incubated under shaking condition at 120 rpm for 5-7 days at room temperature. Lipase enzyme was extracted using centrifugation of culture at 8000 rpm for 5 min and obtained supernatant was used to perform enzymatic assay during 5^{th} and 7^{th} day of incubation.

Solid state fermentation using oil cakes

The solid state fermentation was considered as a common strategy for reducing the enzyme production cost. For the same, effect of various factors was tested using Plackett-Burman design based on the first order model (Plackett and Burman, 1964). The factors optimized and values coded were enlisted in Table 1. Statistically validation of the model was also done using Design Expert 9.0 software. Significant parameters identified from Plackett-Burman designs were selected for Box-Behnken design to obtain response surface curve for the enzyme production. Each factor was studies at two different levels (- 1 and +1) (Table 4). A set of 17 experiments were carried out.

Kinetics of lipase enzyme

Kinetics of Lipase enzyme activity was studied by plotting a Lineweaver- Burk (LB) Plot**.** K_m and V_{max} of the enzyme was predicted. Effect of different solvents, oxidizing and reducing agents on enzyme activity was also verified and the residual activity was calculated. For understanding the hydrolyzing potential of complex oils, the obtained crude lipase enzyme was used to treat olive oil. The enzyme concentrations were varied and incubated at room temperature and after 1 hr the enzyme was inactivated at 100°C. The release of free fatty acids was quantified using standard method (Kamini *et al.,* 2000).

Results and Discussion

Screening and identification of lipolytic fungi

Among 12 fungal isolates screened for lipolytic activity, MF-1 was found to exhibit maximum activity in plate screening with olive oil as a substrate. Release of fatty acids changed the medium pH towards acidic and

plate to yellow colour indicating lipase producing ability of the isolate**.** Secondary screening i.e., lipase assay also revealed that MF-1was the most potent lipolytic fungi. Based on the colony morphology and by microscopic observation of the LCB mount, MF1 was identified as *Aspergillus sp.* MF-1 was identified as *Aspergillus japonicus* through the chromatogram analysis and Blastn analysis of the sequence (D1/D2 region LSU). Further the sequence was submitted in GenBank and an accession number was obtained as KF922321.

Hydrolysis of oil substrates

Hydrolysis of olive oil as a substrate was tested based on the lipase enzyme produced. Varying enzyme dosage (v/v) was used to treat the substrate and free fatty acids released on hydrolysis were estimated. There was an increase in the free fatty acids released with increasing enzyme dosage**.** The potential of hydrolyzing ability of complex hydrocarbons could be exploited in treatment of oil rich effluent and as well as for the biodiesel production using complex natural oil sources. The decrease of hydrolysis beyond a period of 90 min could be due to fatty acids which could inhibit lipase activity (Goswami *et al.,* 2012).

Effluent for lipase production

The utilization of ayurvedic oil effluent for lipase production was evaluated using three different concentrations of effluent (25%, 50% and 100%). The Lipase production was evaluated following 5^{th} and 7^{th} day of incubation. In 100% effluent, there was no significant fungal growth and lipase activity was observed. In case of 25 and 50% effluent, there was fungal biomass and lipase production. However, a maximum of 153.2 U/ml of lipase activity was achieved on 5^{th} day in 25% and on 7^{th} day in 50% effluent (Figure 3). Hence, the ayurvedic oil effluent could be utilized as a support medium along with production medium to achieve maximum lipase production using the *A. japonicus* MF-1. Salgado *et al.,* (2014) utilized two-phase oil mill waste, a major waste of olive mill industry and achieved a maximum lipase activity of 18.67 U/g by *A.ibericu*. However, this was obtained by supplementation of 0.073g urea/g and 25% EGM along with the effluent which stimulated the production.

Solid state fermentation using GOC

Microbial lipases are produced mostly by submerged culture (Ito *et al.,* 2001), but solid state fermentation methods can also be used (Chisti, 1999). Rivera-Munoz *et al.,* (1991) verified the superiority of SSF over Submerged fermentation using *Penicillium candidum* for lipase production. There were many reports on the lipase production by *Aspergillus sp*. on SSF using oil cakes. The groundnut oil cake (GOC) was used as a substrate for lipase production using *A. japonicus* MF-1. Kamini *et al.,* (2000) used gingelly oil cakes for lipase production using *Aspergillus niger*. Similarly, Christen *et al.,* (1995) obtained lipase from *Rhizopus delemar* grown on a polymeric resin. Kumar and Ray (2014) used Plackett-Burman design for optimization of lipase production using a *Pseudomonas sp.*AKM-L5. For optimization of the most favourable conditions, Plackett-Burman design was used. The tested variables and coded values were mentioned in table 1. Maximum lipase activity of 266 U/g was achieved during solid state fermentation conditions. The predicted model had R^2 value of 99.05% and predicted response of 90.88%. The model indicated that a high inoculum percentage of 10 with incubation temperature of 55°C for 168 hrs of incubation could achieve maximum lipase activity using GOC as substrate even in lowest moisture percentage of 20. The regression model for the optimized condition was mentioned as Equation 1.

Table.1 Variable used in Plackett-Burman model for optimization of lipase production under SSF with high and lower values of the factors tested

Code	Variables	Units	Low	High
			-1	+1
A	Moisture	%	20	60
B	Temperature	°C	28	55
C	pH		5	7
D	Inoculum	% (v/w)	3	10
E	Incubation	Hours	72	168
F	KH_2PO_4	g/L	2	4
G	Peptone	g/L	5	10
H	Urea	g/L	1	3
I	Olive oil	ml/L	10	50
J	KCl	g/L	2	5

Table.2 Lipase activity at various runs of Plackett-Burman design using *A. japonicus* MF-1

Run	A	B	C	D	E	F	G	H	I	J	K	Lipase Activity (U/g)	
												Experimental	Predicted
1	-1	-1	1	-1	1	1	-1	1	1	1	-1	106.4	106
2	-1	1	1	-1	1	1	1	-1	-1	-1	1	53.2	54
3	-1	1	-1	1	1	-1	1	1	1	-1	-1	266	265
4	-1	1	1	1	-1	-1	-1	1	-1	1	1	26.6	26
5	1	-1	1	1	1	-1	-1	-1	1	-1	1	26.6	27
6	1	-1	1	1	-1	1	1	1	-1	-1	-1	79.8	80
7	-1	-1	-1	1	-1	1	1	-1	1	1	1	79.8	79
8	1	1	1	-1	-1	-1	1	-1	1	1	-1	26.6	25
9	1	1	-1	-1	-1	1	-1	1	1	-1	1	133	133
10	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	26.6	27
11	1	-1	-1	-1	1	-1	1	1	-1	1	1	159.6	160
12	1	1	-1	1	1	1	-1	-1	-1	1	-1	106.4	105

[A: Moisture (%); B: Incubation temperature (°C); C: pH; D: Inoculation %; E: Incubation time (hours); F: KH_2PO_4 (g/l); G: Peptone (g/l); H: Urea (g/l); I: Olive oil (ml/l); J: KCl (mg/l); K: Dummy variable]

Table.3 Analysis of Variance (ANOVA) for the factorial model on Lipase production by *Aspergillus japonicus* MF-1 through SSF utilizing groundnut oil cake (GOC) as substrate

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	Status
Model	53774.56	8	6721.82	9.771429	0.043607	significant
B-Temperature	1474.083	1	1474.083	2.142857	0.239443	-
C-pH	17040.4	1	17040.4	24.77143	0.015587	*
D-Inoculum	530.67	1	530.67	0.771429	0.444438	-
E-Incubation	9964.803	1	9964.803	14.48571	0.031874	*
F-KH_2PO_4	58.96333	1	58.96333	0.085714	0.78878	-
G-Peptone	4776.03	1	4776.03	6.942857	0.077994	-
H-Urea	17040.4	1	17040.4	24.77143	0.015587	*
J-Olive oil	2889.203	1	2889.203	4.2	0.132842	-
Residual	2063.717	3	687.9056			
Total	55838.28	11				

[* Significant factors based on Prob > F values]

Table.4 Variables used for the Lipase enzyme optimization using *A. japonicus* MF-1 based on Box-Behnken design

Variables	Units	Low	High
		-1	1
pH		3	9
Incubation time	hrs	72	168
Urea	g/l	1	3

Table.5 Lipase activity obtained from different runs of the Box-Behnken experimental design using *A. japonicus* MF-1

Runs	Lipase Activity (U/g)	
	Experimental	Predicted
1	741	740
2	684	684
3	684	683
4	684	682.5
5	627	628
6	684	683
7	570	569
8	684	684
9	513	514
10	570	569
11	741	742
12	627	627
13	684	682
14	627	628
15	513	513
16	231	232
17	721	721

Table.6 Comparison of Lipase production by *Aspergillus* sp.

Organisms	Substrate Used	Maximum Lipase activity	References
Aspergillus japonicus MTCC 1975 Mutant ANT 4	Production medium	20.6 U/ml	**[32]**
Aspergillus niger 11T5	Wheat bran	153.4 U/gdm	**[33]**
A.japonicus	Malt extract, Wheat mill bran, Soy flour, and Whey	177.5 U/ml	**[34]**
A.japonicus LAB01	Basal medium	199.5 U/ml	**[35]**
A. candidus URM 5611	Almond bran licuri	395 U/gd/s	**[36]**
A.japonicus MF-1	Groundnut oil cake	266 U/g	Present Work

Table.7 ANOVA for the influence of the selected variable on xylanase production using *Aspergillus japonicus* MF- 1 based on Box-Behnken experimental design

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	Status
Model	1.764E+005	7	25197.17	3.39	0.0462	significant
A-pH	4560.13	1	4560.13	0.61	0.4537	
B-Incubation time	90738.00	1	90738.00	12.20	0.0068	
C-Urea	12720.13	1	12720.13	1.71	0.2233	
BC	19881.00	1	19881.00	2.67	0.1365	
A^2	24915.60	1	24915.60	3.35	0.1004	
B^2	15069.60	1	15069.60	2.03	0.1883	
C^2	10769.81	1	10769.81	1.45	0.2595	
Residual	66925.70	9	7436.19			
Lack of Fit	44832.50	5	8966.50	1.62	0.3296	not significant
Pure Error	22093.20	4	5523.30			
Cor Total	2.433E+005	16				

Table.8 Effect of solvents on activity of lipase from *A. japonicus* MF-1

Organic Solvents	Concentrations (%)	Relative Activity (%)
Control	0	100
Acetone	5	100
	10	56.4
	15	32
Ethanol	5	96
	10	92.4
	15	78
Methanol	5	100
	10	140
	15	173
Hexane	5	60
	10	32
	15	27.5
DMSO	5	80
	10	100
	15	110

Table.9 Effect of oxidizing and reducing agents on activity of lipase produced by *A. japonicus* MF-1

Oxidation/reducing agents	Concentrations (%)	Relative Activity (%)
Control	0	100
H_2O_2	5	94.5
	10	80
	15	40
β -Mercaptoethanol	5	310
	10	290
	15	380

Figure.1 Neighbour Joining tree showing evolutionary relationship among other *Aspergillus sp*. and the present isolate *A. japonicus* MF-1

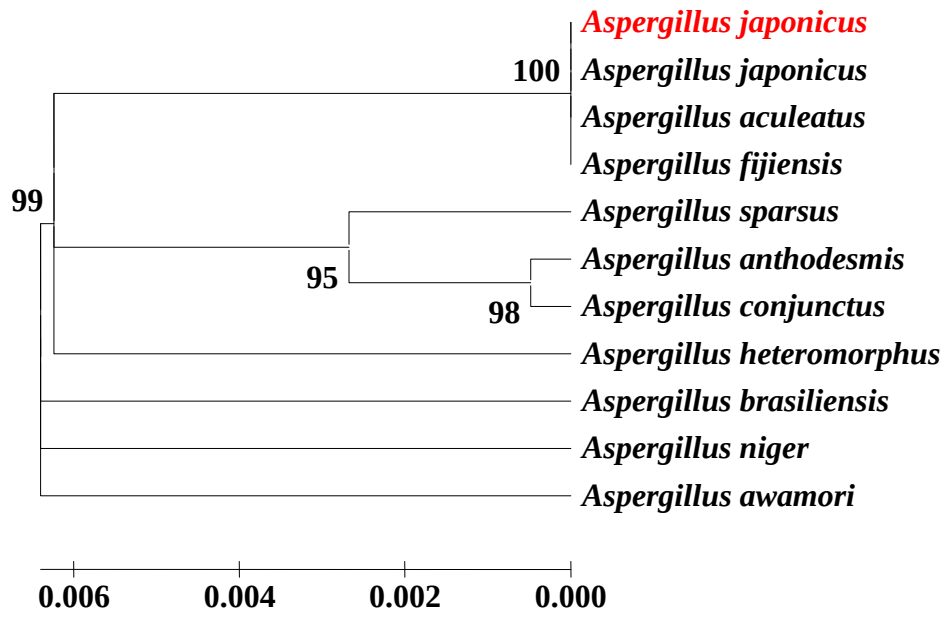


Figure.2 Kinetics showing hydrolysis of olive oil substrate using *A. japonicus* MF-1 lipase and release of free fatty acids

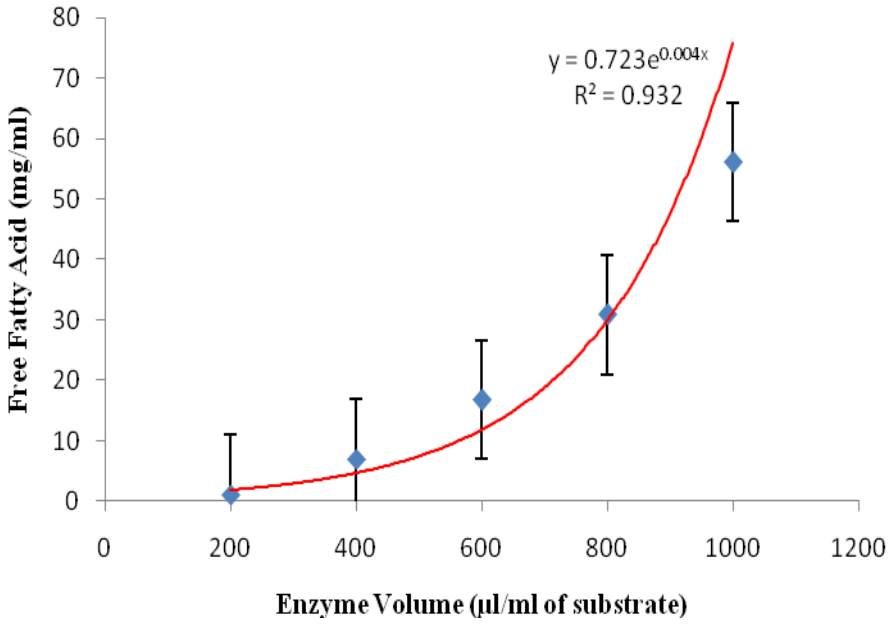


Figure.3 Medicinal oil effluent based medium for lipase production using *A.japonicus* MF-1 [MM- Minimal medium; E- Enzyme dosage]

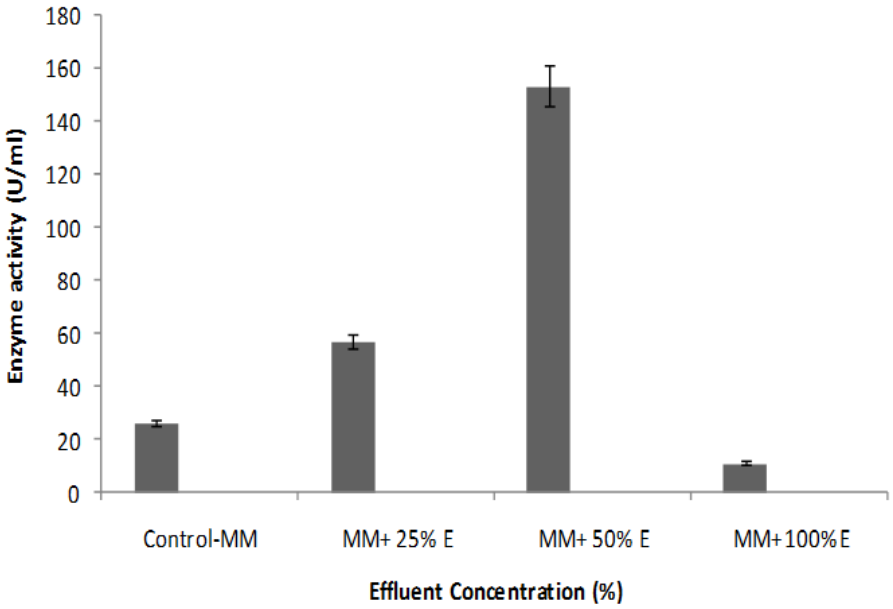


Figure.4 Contour plots showing effect of selected parameters during lipase optimization through SSF using *A. japonicus* MF-1 *based on* Plackett-Burman method

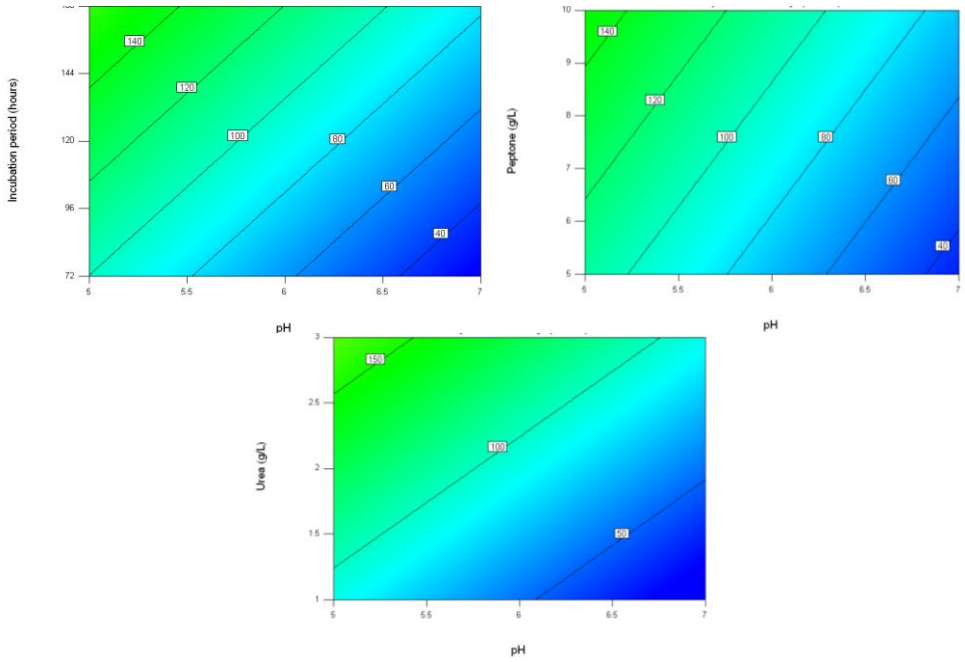
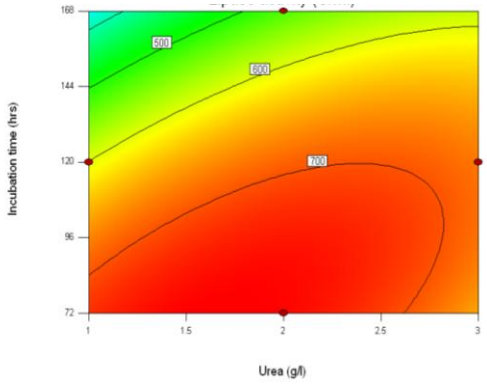


Figure.5 Contour plots showing effect of significant parameters during lipase optimization through SSF using *A. japonicus* MF-1 based on Box-Behnken method



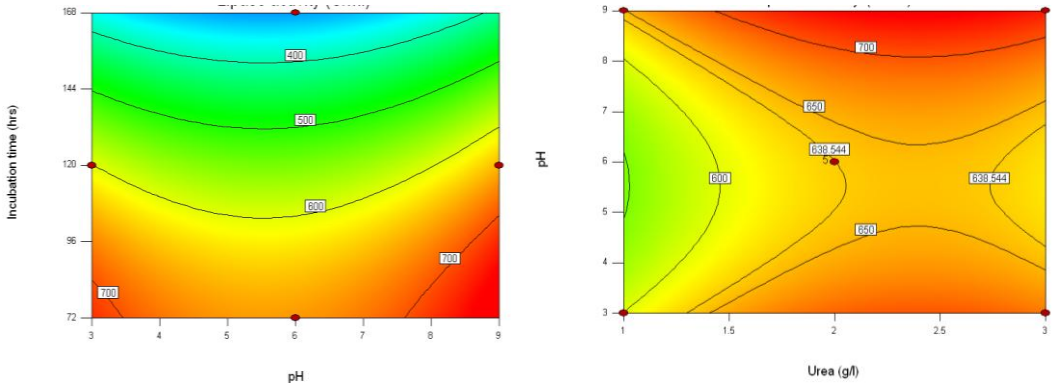
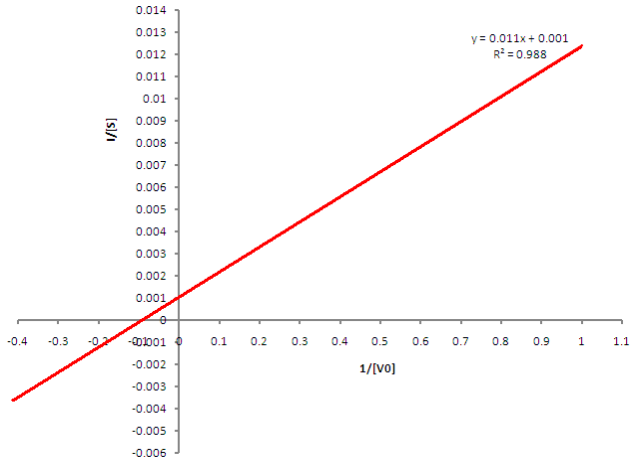
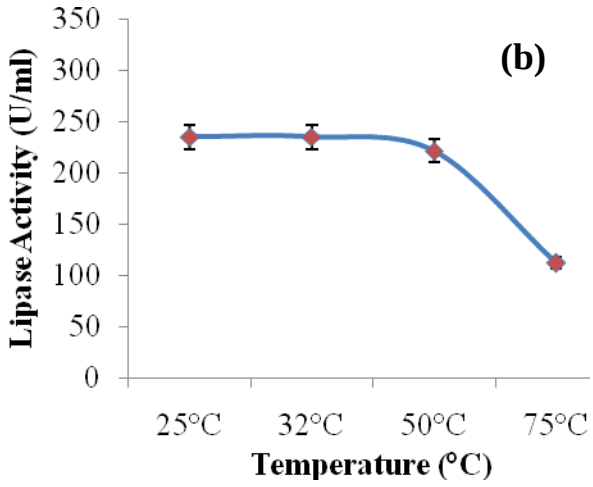


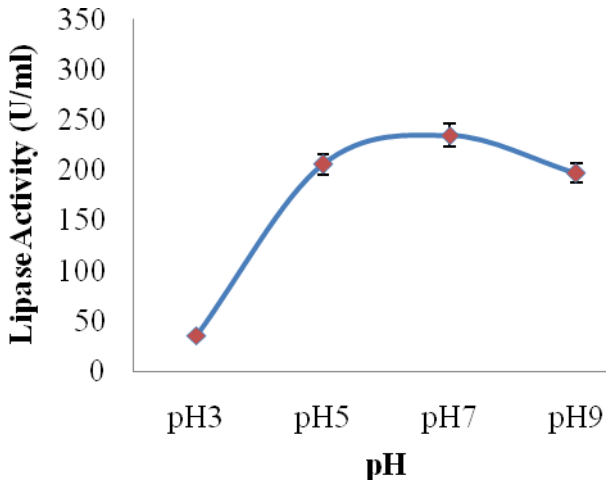
Figure.6 Kinetics and Physico-chemical property of Lipase from *A. japonicus* MF-1
a) Lineweaver- Burk plot showing kinetics of lipase enzyme
b) Optimum temperature and c) optimum pH





(b)

(c)



There were variations between actual and predicted model with a difference in R^2 values. The actual response and the predicted values were enlisted in table 2. Based on the ANOVA results, moisture percentage was identified as the most influential factor from this study (Table 3). Figure 4 depicts the 3D response graphs of the significant factors on lipase activity.

$$\text{Lipase activity (U/ml)} = 192.996 + 20.9205 * A + 35.0078 * E - 38.3412 * F + 43.8505 * H - 64.728 * J + 46.8505 * K$$

Equation 1

Where Y = Lipase activity (U/ml)

Behnken Optimization Design for Lipase production using *Aspergillus japonicus* MF-1

In Lipase optimization, medium pH, incubation time and urea concentration was varied during Box-Behnken experimental design. It was found that a maximum lipase activity of 741 U/g was achieved from the model (Table 5). The predicted model was found to be significant with a R^2 value of 0.7249 (Table 7). The quadratic regression equation describing the model was written as Equation 2. Response surface curve and contour plots revealed that pH and urea was the most significant factor for lipase production with high response at high and low variables tested. Similarly, with high urea concentration and towards alkaline pH, minimum incubation time was required for maximum lipase production (Figure 5). Lanka and Latha (2015) optimized lipase production using *Emericella nidulans* NFCCI 3643based on Box-Behnken design which resulted in an enzyme activity of 409.723 U/ml, which was 2.76-fold higher than the value obtained through traditional OFAT (one factor at a time) method. However, a high lipase activity was achieved using the *A. japonicus* MF-1 compared to other *Aspergillus* sp. (Table 6).

Lipase activity (U/ml) = +861.14375 - 94.60833 * pH +1.07552 * Incubation time +65.92500 * Urea +1.46875 * Incubation time * Urea +8.54722 * pH^2-0.025966 * Incubation $time^2$ -50.57500 * $Urea^2$

Equation 2

Enzyme kinetics of lipase

The K_m and V_{max} values were determined using LB plot constructed (Figure 6a). The K_m and V_{max} values of the *A. japonicus* MF-1 were found to be 11 mML^{-1} and $1Mmin^{-1}$ respectively. The effect of various solvents on the residual activity of the lipase enzyme was calculated and tabulated in table 8. The effect

of various organic solvents on lipase activity was studied earlier. DMSO and methanol could enhance the lipase residual activity which was higher than previous reports. Kamini *et al.,* (2000) achieved about a maximum of about 124% residual activity in the presence of 10% DMSO. Similarly, Akkaya and Yenidunya (2012) reported an increase of 260% and 170% of residual activity with some other solvents like 2-3, epoxypropyl methacrylates and hydroxymethyl methacrylate (HEMA). Similarly, the effect of oxidizing and reducing agents were also evaluated (Table 9).

The reducing agent mercaptoethanol could enhance the residual lipolytic activity; however the oxidizing agent lowered the activity. This was contradicting the early reports which might be due to the high concentration of H_2O_2 used in the present study. Mander *et al.,* (2014) reported the enhancing effect of lipase residual activity of reducing agent like mercaptoethanol and H_2O_2 an oxidizing agents. The optimum Lipase activity was observed at pH 7 and between temperature ranges of 32- 50°C (Figure 6b and c). Mahadik *et al.,* (2002) produced acidic lipase from *Aspergillus niger* through solid state fermentation.

In conclusion lipase production using *A. japonicus* MF-1 utilizing ground nut oil cake as a substrate in solid state fermentation was found to be promising and economic. The lipase enzyme obtained exhibited higher residual activity in the presence of oxidizing and reducing agents. Submerged fermentation utilizing medicinal oil effluent for lipase production open up new scope of using the isolate in bio-remediation of oil rich industrial effluents through hydrolysis of such complex oils and further prospected its utility as a bio-fuel.

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