SOME OPTIMAL PARAMETERS FOR FUNGAL LIPASE ACTIVITY

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Abstract

Lipolytic fungi were isolated from different sources. Soil sample was collected from the car workshop as fuel oil contaminated soil, Thuwana Township, Yangon Region, Myanmar. Other samples were collected from pork sausage and cheese. Fungal strains were directly isolated from 2 different sources. Diluted soil (concentration - 10^{-3}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}) was used to culture fungi. Fungal strains were cultured on Potato Dextrose Agar (PDA) medium. Lipolytic fungi were screened using Tributyrin Agar (TBA) medium. The isolated fungi were identified by their pure colony morphology and spore formation according to the references. In the present study, three different types of lipolytic fungi were observed from three different sources. Some optimal parameters such as fermentation period, substrate, incubation temperature and initial pH were investigated for lipase activity. Lipase from *Aspergillus* sp. (1) (isolated from pork sausage source) showed the maximum lipase activity at pH 6.0 and 40ºC. Lipase produced from *Penicillium* sp. (isolated from cheese source) exhibited the greatest lipase activity at 45 ºC and pH 8.0, while the highest lipase activity from *Aspergillus* sp. (18) (isolated from fuel oil contaminated soil) was at 50 ºC and pH 8.5.

Keywords: Lipolytic fungi, Lipase, Parameter

Introduction

Lipase enzymes (Triacylglycerol acyl-hydrolase; EC 3.1.1.3) hydrolyze triacylglycerols which are the major constituents of fats and oils. Lipases and esterases catalyze both hydrolysis and synthesis of ester (Griebeler *et al.*, 2011). Lipase enzymes hydrolyze the ester bonds of insoluble substrates in water at the substrate-water interface (Colla *et al.*, 2015).

Fungi are the best microbial sources for commercial lipase production because these can be easily extracted from fermentation processes in short time. Fungal lipases are high productivity, low costs, safe and easy handling. Lipase production by filamentous fungi mainly depends on various factors such as oil substrates, optimum pH and temperature. Optimum parameters are crucial for the best production of extracellular enzyme (Wadia and Jain, 2017 & Kotogan *et al.*, 2014).

Industrial attention has particularly increased in microbial lipases due to their substrate specificity, stability, and various industrial applications like detergent, food, pharmaceutical, dairy, cosmetic, perfumes, biodiesel, paper, and leather (Shukla and Desai, 2016). Lipases play an important role in numerous industrial applications. It is need to study their characteristics because lipases obtained from different sources may have different properties (Colla *et al.*, 2015).

This study was undertaken to observe the best fermentation period, substrate, and the effect of temperature and pH on the maximum activity of lipase enzyme.

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Materials and Methods

Sample preparation from different sources

1. Collection and isolation of lipolytic fungi from pork sausage (PS)

Small pieces of pork sausage sample were kept in a plastic bowl. Pork sausage in the plastic bowl was incubated for two weeks until fungal growth was observed.

2. Collection and isolation of lipolytic fungi from cheese (CH)

Cheese was incubated in the plastic cup for eight days until fungal growth was observed.

3. Collection and isolation of lipolytic fungi from fuel oil contaminated soil (OS)

Fuel oil contaminated soil sample was taken from the car workshop, Thuwana Township, Yangon Region, Myanmar. Soil sample was dried in the air. A ten-fold dilution series of soil was prepared according to Alexander and Strete (2001).

Cultivation of Fungi

Fungal strains were directly collected from pork sausage, cheese, and diluted soil contaminated with fuel oil. Fungi were cultivated and isolated on Potato Dextrose Agar (PDA: Mash Potato 200 g, Peptone 3 g, Dextrose 20 g, Agar 20 g, Distilled water 1000 mL, pH 6.5 ± 2 according to Atlas, 1993) medium at room temperature for 5 - 7 days old. The pure fungal strains were maintained in test tubes with PDA medium. PDA medium was also used as stock culture medium or sub-culture medium for maintenance of fungus according to Atlas, 1993. All stock cultures were stored at 4 ºC. Chloramphenicol was added for antibacterial activity.

Screening of lipolytic fungi using Tributyrin Agar (TBA) medium

Screening of lipase producing fungi was done using tributyrin as a substrate on agar plates. Two different percentages (0.1 % and 1 %) of Tributyrin were used in this study. Lipolytic fungi were screened using Tributyrin Agar medium with 0.1 % tributyrin (Composition %/mL: Peptone 0.5 g, Yeast extract 0.3 g, Tributyrin (HiMedia) 0.1 mL, Agar 2.0 g, pH 6.0) according to Kotogan *et al.*, (2014) and Griebeler *et al.*, (2011). In addition, Tributyrin Agar (TBA) medium with 1 % tributyrin (composition %/mL: Peptone 0.5 g, Yeast extract 0.3 g, Agar 2.0 g, Tributyrin (HiMedia) 1.0 mL, pH 7.5 ± 0.2) was also used for screening of lipolytic fungi according to Wadia and Jain (2017). Clear hydrolytic halo regions occurred around colonies, it indicated that lipase enzyme was produced. All the isolated fungal cultures were inoculated on TBA plates and incubated at room temperature for 2 - 17 days.

Identification of lipolytic fungi

Fungi were identified according to Barnett (1960) and Dube (1983).

Preparation of inoculum and optimization of culture medium

Four different media were prepared for production of lipase by submerged fermentation. Seven days old fungal culture, which was already grown in Czapek-Dox Agar slant test tube, was used for preparation of inoculum. The spores were scratched with sterile inoculating loop and mixed with 10 ml of sterile distilled water in a tube. Spore suspension was transferred into 500 ml conical flask containing 200 ml of fermentation medium (Pandey *et al.*, 2016). The optimal density of fermentation broth was taken and examined the lipase production in different time intervals such as 1 day, 2 day, 3 day, 4 day, 5 day, 6 day, 7 day, etc. The composition of four different fermentation

media (g/L) was as; medium 1 (M1) (Glucose 10 g, Peptone 20 g, NaCl 5 g, Yeast extract 5 g, pH 6.0 ± 0.2 and Coconut oil 10 mL) (Prabhakar *et al.*, 2002); M2 (KH_2PO_4 0.25 g, KCl 0.5 g, $MgSO_4.7H_2O$ 0.5 g, Peptone 8.0 g, Glucose 8.0 g, pH 8 ± 0.2 and Sunflower oil 15 ml) (Pandey *et al.*, 2016); M3 (KNO_3 3.0 g, KH_2PHO_4 1.0 g, $MgSO_4.7H_2O$ 1.0 g, pH 6.5 ± 0.2 and Olive oil 20 ml) (Brooks and Asamudo, 2011); M4 (KNO_3 2.5 g, KH_2PO_4 1 g, $MgSO_4$ 0.5g, NaCl 5 g, pH 8 ± 0.2 and Olive oil 15 ml) (Lanka and Trinkle, 2017).

Extraction of enzyme from fermentation broth

All media were examined by taking optimal densities on 1 day, 2 day, 3 day, 4 day, 5 day, 6 day and 7 day of fermentation to observe the best medium for lipase production. The filtrates were centrifuged at 10,000 rpm, 4˚C for 10 minutes to obtain supernatant. The clear supernatant was considered as crude enzyme. The resulting supernatant was evaluated for lipase activity by using p-nitrophenyl palmitate (pNPP, Sigma) as a substrate as described by Winkler and Stuckmann, 1979 (Massadeh and Sabra, 2011, Pandey *et al.*, 2016, and Rodrigues *et al.*,2016).

Lipase activity assay

The extracellular lipase activity was determined by using p-nitrophenyl palmitate (p-NPP) (Sigma, USA) as substrate according to Winkler & Stuckmann (1979). The pNPP substrate solution was prepared by freshly mixing solution A (3 mg of pNPP in 1 ml of isopropanol) with solution B (10 mg of gum Arabic and 40 ml of Triton X-100 in 9 ml of Tris-HCl buffer, pH 8.0) while stirring until all was dissolved. Freshly prepared 1 ml of p-NPP solution was incubated in a water bath at 37ºC for 10 minutes. After 10 min, 0.5ml of crude enzyme sample and 0.5 ml distilled water was added and the reaction mixture was further kept in the water bath for 30 min at 35 - 37ºC. After that, the enzymatic reaction was stopped by adding 0.1 ml of 100 mM $CaCl_2.2H_2O$. The formation of yellow color due to release of p-nitrophenol indicated lipase activity. The absorbance of yellow color was measured by spectrophotometry at 410 nm against a control without enzyme (Massadeh and Sabra (2011), Pandey *et al. (*2016), Rodrigues *et al.* (2016)). The concentration of liberated yellow color compound (p-nitrophenol) in the reaction mixture was determined by using standard curve of p-nitrophenol (4 to 20 µg ml^{-1} in 0.05 M Tris HCl buffer, pH-8.0) (Kanwar *et al.*, 2005). One unit (U) of lipase activity was defined as micromole (µM) of p-nitrophenol liberated from the hydrolysis of p-nitrophenyl ester by one ml of soluble enzyme per minute under standard assay conditions.

Preparation of p-nitrophenol standard curve

Standard curve of p-nitrophenol was prepared using the concentration range of p-nitrophenol (4, 8, 12, 16 and 20 µg ml^{-1}) in 0.05 M Tris HCl buffer, pH-8.0 according to Kanwar *et al.*, 2005 and www.Shodhganga.inflibnet. ac>bitstream.com.

Effect of different lipid substrates on lipase activity

The substrate of fermentation medium was replaced with various lipid substrates such as coconut oil, sunflower oil, olive oil, peanut oil and lard (rendered pig fat) (Basheer, 2007 and Sirisha *et al.*, 2010).

Effect of Temperature on lipase activity

The effect of temperature on lipase activity was investigated at temperatures ranging from 25 to 50˚C by keeping the remaining parameters same (Sirisha *et al.*, 2010, Basheer, 2007 and Niaz *et al.*, 2014).

Effect of pH on lipase activity

The optimum pH for lipase activity was examined by varying from pH 5.0 to 9.0. The remaining parameters were unaltered (Basheer, 2007 and Sirisha *et al.*, 2010).

Results

Isolation of lipolytic fungi from different sources

Lipolytic fungi were isolated from different sources. In this study, some optimal parameters of three different types of lipolytic fungi as shown in Table 1 such as *Aspergillus* sp. (1) (was selected from 3 strains) from pork sausage (Figure 1), *Penicillium* sp. (was selected among 5 strains) from cheese (Figure 2), and *Aspergillus* sp. (18) (was selected from 8 strains) from fuel oil contaminated soil (Figure 3) were observed. Each lipolytic fungus was identified based on their characters of pure colony morphology and spore formation according to Barnett (1960) and Dube (1983).

Table 1 Lipolytic fungi from different sources

No.	Fungal sources	Lipase producing fungi	Code of isolated strains	Showed clear zone (Halo region)	
				0.1%TBA	1% TBA
1.	Pork sausage	*Aspergillus* sp. (1)	PS-1	After 2-5 days	After 5- 17 days
2.	Cheese	*Penicillium* sp.	CH-1	‖	‖
3.	Fuel oil contaminated soil	*Aspergillus* sp. (18)	OS-8	‖	‖

Identification of isolated lipolytic fungi

Characteristics of mycelium and spore formation of *Aspergillus* sp. isolated from Pork Sausage (PS)

Aspergillus sp. (1) colony was yellow color inside and white color periphery. Mycelia were scattered in culture.

(A) **(B)** **(C)** **(D)**

Figure 1 Lipolytic fungus *Aspergillus* sp. (1) from pork sausage

(A) Fungal growth on pork sausage (B) Fungal colony (5 - 7 days old) (yellow color inside and white color periphery) (PS) (C) Clear zone (halo) around fungal colony (2 days old) on 1 % TBA medium (D) Micrograph of *Aspergillus* sp. (1) (X 200)

Characteristics of mycelium and spore formation of *Penicillium* sp. isolated from cheese (CH)

Penicillium sp. colony was green color inside and white color periphery. Mycelia were scattered in culture.

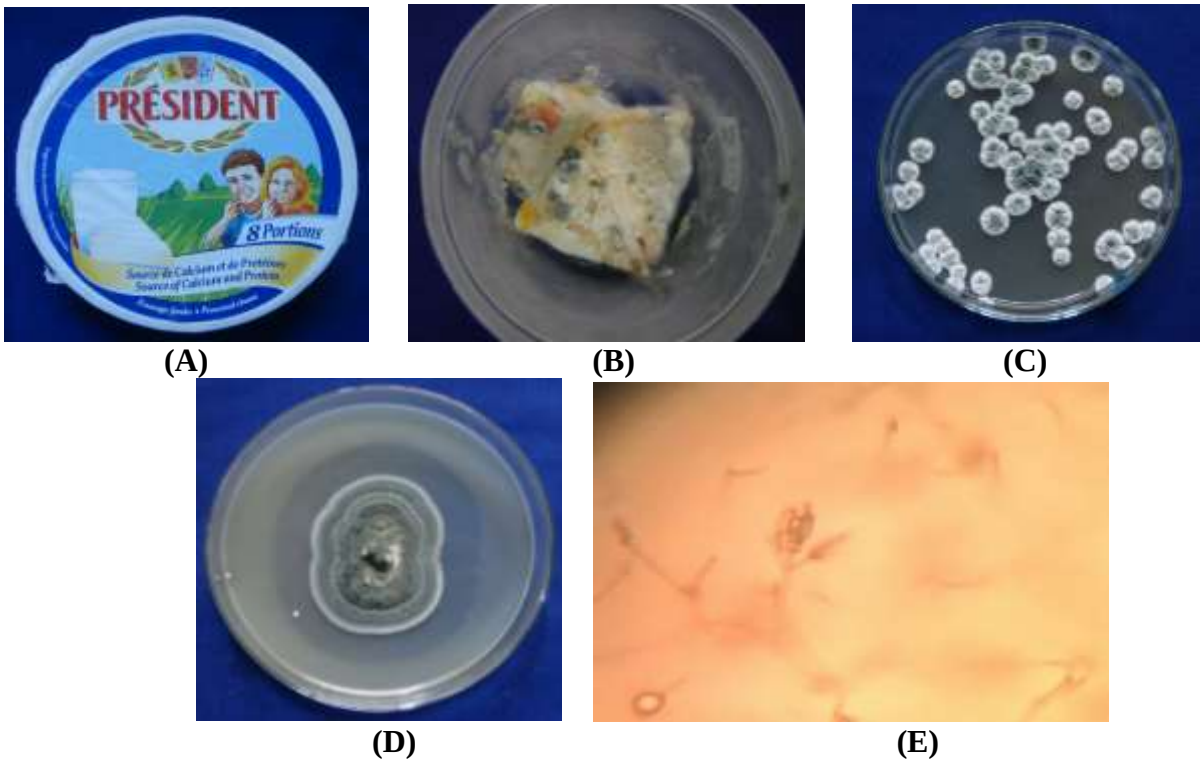


Figure 2 Lipolytic fungus *Penicillium* sp. from cheese

(A) Collected cheese source (B) Fungal growth on cheese (C) Pure fungal colony (5 - 7 days old) (green color inside and white color periphery) (CH - 1) (D) Clear zone (halo) around fungal colony (12 days old) on 1 % TBA medium (E) Micrograph of *Penicillium* sp. (X 400)

Characteristics of mycelium and spore formation of *Aspergillus* sp. isolated from fuel oil contaminated soil (OS)

Aspergillus sp. (18) colony was black color inside and white color periphery. Mycelia were scattered in culture.

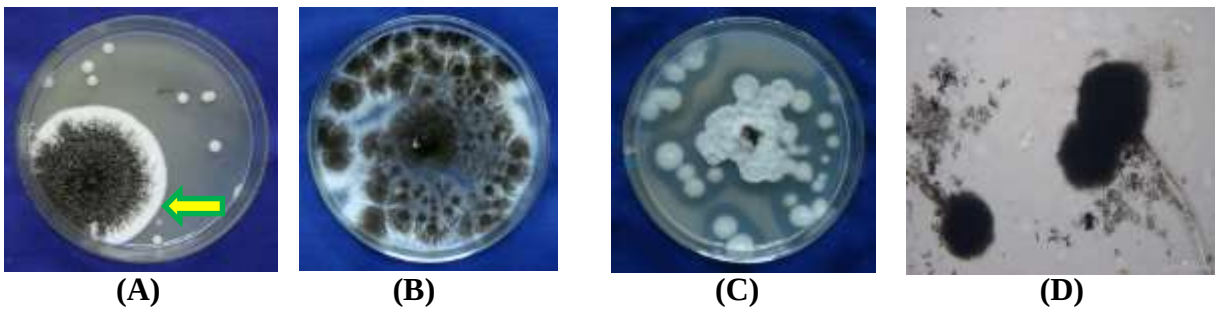


Figure 3 Lipolytic fungus *Aspergillus* sp. (18) from fuel oil contaminated soil

(A) Fungus isolated from soil sample (10^{-8}) (B) Pure fungal colony (5 - 7 days old) (black color inside and white color periphery) from soil sample (10^{-8}) (C) Clear zone (halo) around fungal colony (6 days old) on 1% TBA medium (D) Micrograph of *Aspergillus* sp. (18) (X 200)

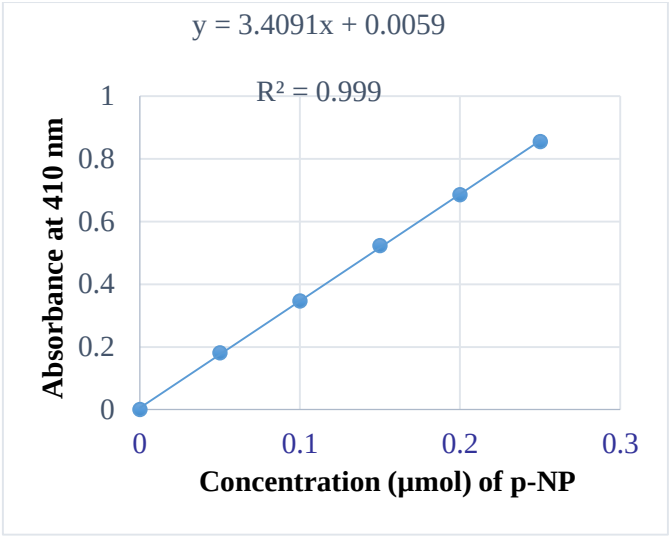


Figure 4 p-nitrophenol (p-NP) standard curve

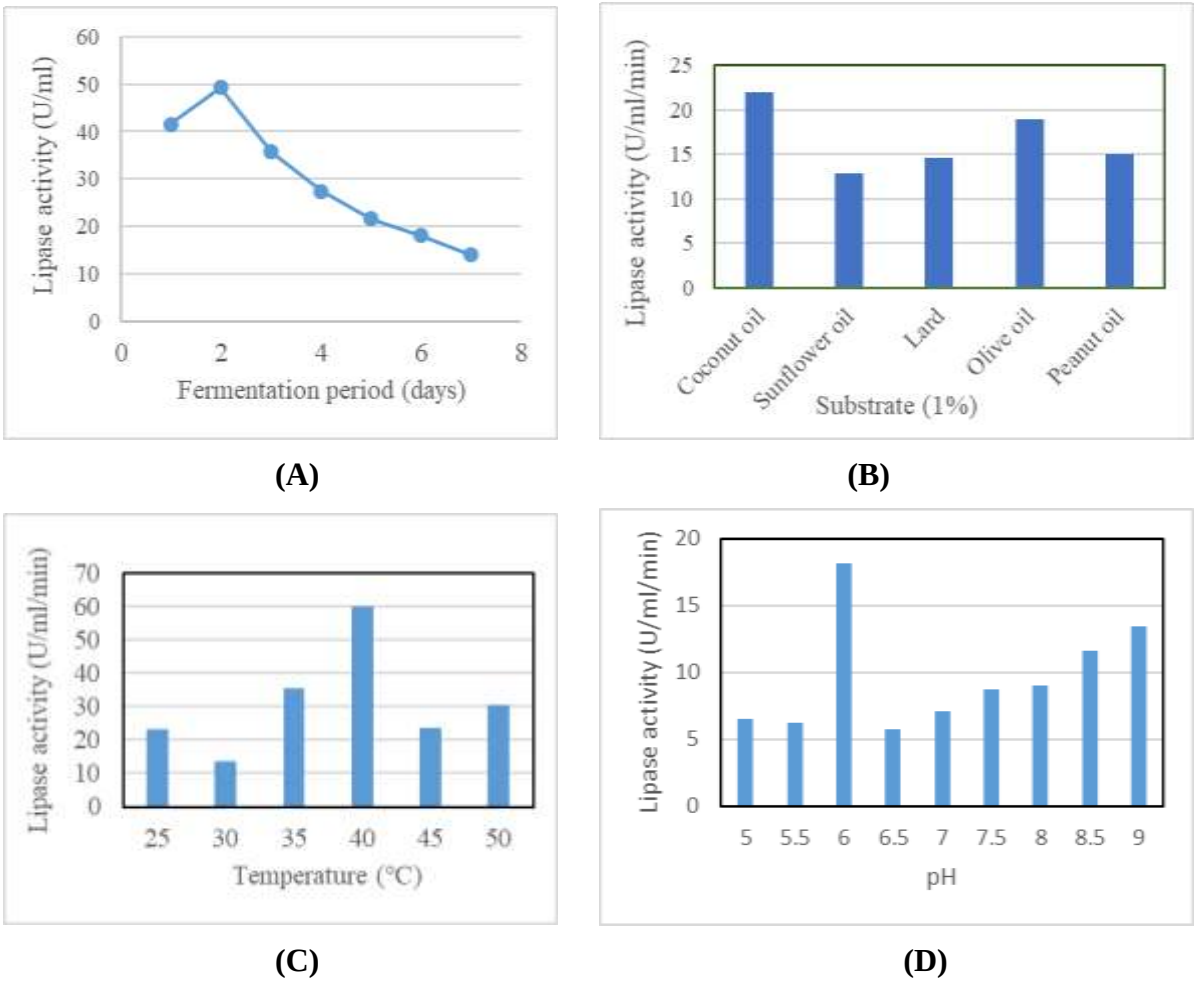
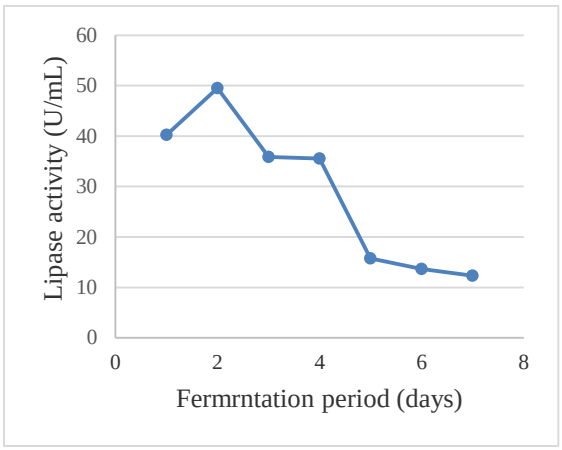
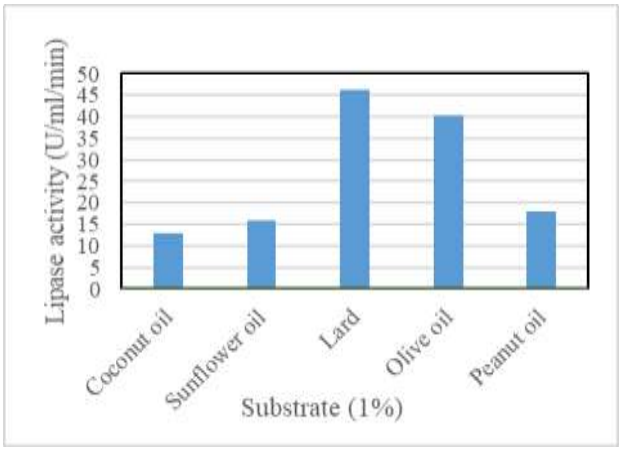


Figure 5 Effect of incubation period, substrate, temperature and pH on lipase activity by *Aspergillus* sp. (1) from pork sausage

(A) Effect of incubation period on lipase activity, (B) Effect of different oil substrates on lipase activity, (C) Impact of temperature on lipase activity, (D) Effect of pH on lipase activity



(A)



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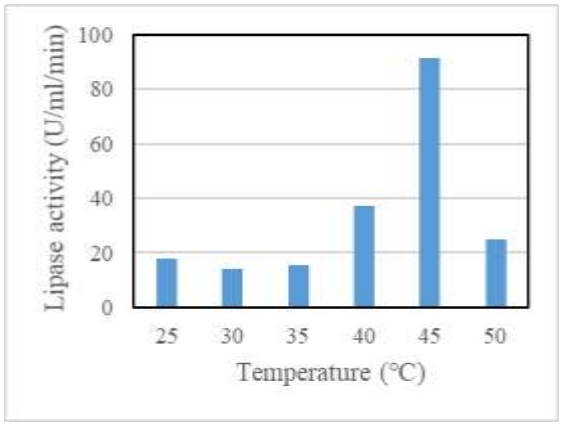

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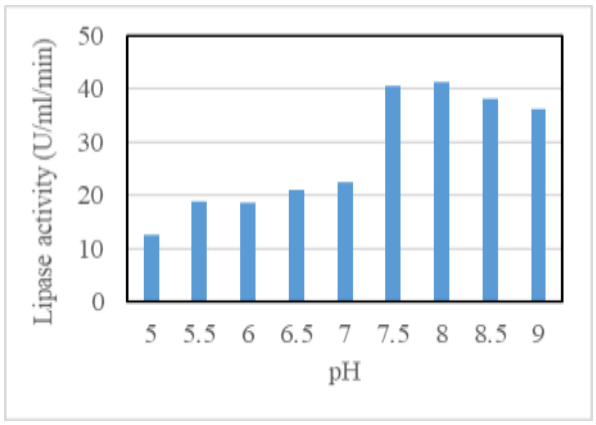
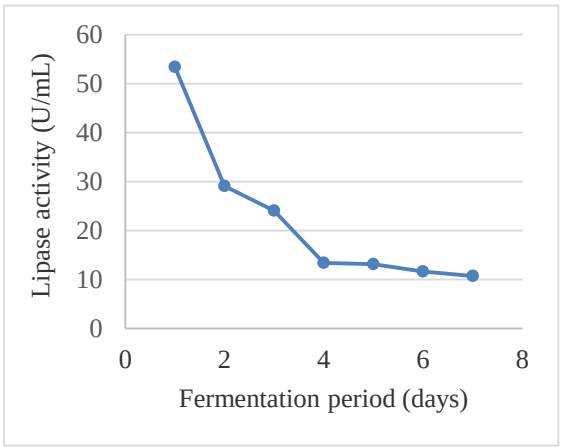


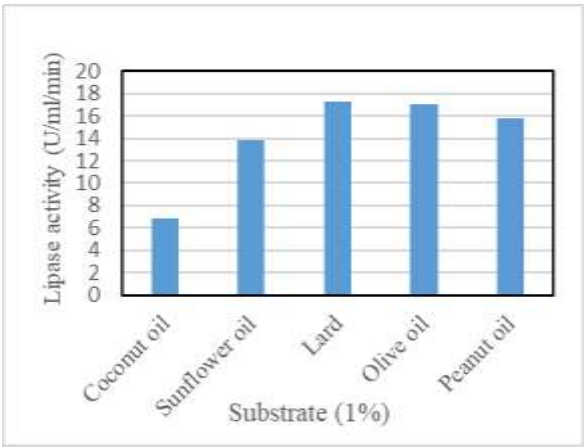
(D)

Figure 6 Effect of incubation period, substrate, temperature and pH on lipase activity by Penicillium sp. from cheese

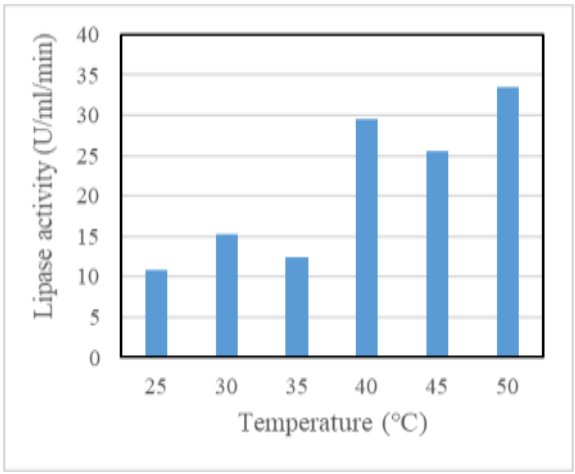
(A) Effect of fermentation period on lipase activity, (B) Effect of various lipid substrates on lipase activity, (C) Impact of temperature on lipase activity, (D) Effect of pH on lipase activity



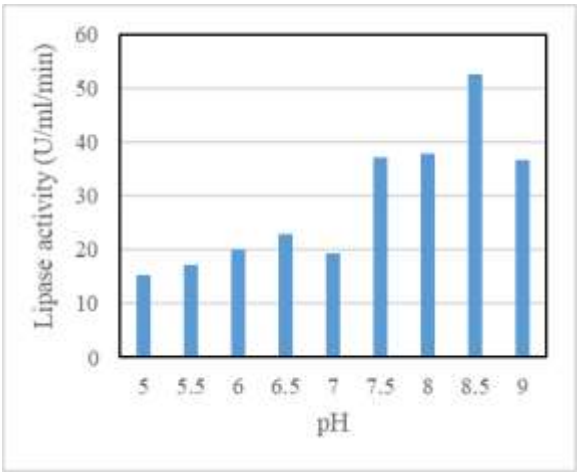
(A)



(B)



(C)



(D)

Figure 7 Effect of incubation period, substrate, temperature and pH on lipase activity by *Aspergillus* sp. (18) from fuel oil contaminated soil

(A) Effect of fermentation period on lipase activity, (B) Impact of different lipid substrates on lipase activity, (C) Effect of temperature on lipase activity, (D) Impact of pH on lipase activity

Discussion and Conclusion

In this research work, three different lipolytic fungi were selected and inoculated through four different submerged fermentation media to obtain the best lipase production. Some optimal experimental conditions such as effect of incubation period, substrate, temperature and pH were studied for production of lipase enzyme after medium optimization. The lipase activity was estimated by spectrophotometric method using p-NPP substrate solution.

In the present investigation, isolated *Aspergillus* sp. (1) from pork sausage and isolated *Penicillium* sp. from cheese produced the maximal lipase activity on second day. Lipase from *Aspergillus* sp. (1) exhibited the highest activity at 40 °C. Brooks and Asamudo (2011) reported that optimum temperature of lipase activity was 40 °C for *Aspergillus niger* AC-5 and 45 °C for *A. niger* AC-7 which were isolated from contaminated body creams.

In this study, *Aspergillus* sp. (1) produced the greatest lipase activity (18.13 U/ml/min) at pH 6.0. Prabhakar *et al.,* (2002) reported that *Aspergillus japanicus* and isolated *Aspergillus* sp. from contaminated ghee showed the best lipase activity at pH 6.0. The highest activity of crude lipase from *A. japanicus* was 34 U/ml and the isolated *Aspergillus* sp. was 36 U/ml under the optimized conditions.

In the present study, *Penicillium* sp. and isolated *Aspergillus* sp. (18) from fuel oil contaminated soil showed maximum activity at 45 °C and 50 °C. Bakir and Metin (2017) reported that lipase enzyme which shows maximum activity at 45, 50 and 55 °C may be useful for various processes such as detergent, leather, medical, cosmetic, textile and food industries.

In this study, lipase from *Aspergillus* sp. (18) showed the maximum activity at pH 8.5 while *Penicillium* sp. produced the best lipase activity at pH 8. The present results proved that the enzyme under investigation is an alkaline lipase. Colla *et al.* (2015) stated that a lipase obtained from *A. fumigatus* presented optimum pH of 8.5.

In the present study, lipase produced by *Penicillium* sp. through submerged fermentation showed the maximum activities at 45 °C and pH 8.0, while lipase from *Aspergillus* sp. (18)

exhibited at 50 °C and pH 8.5. The pH was higher in alkaline pH. Lipase produced through Smf with *Aspergillus* sp. (1) had optimum temperature and pH at 40 °C and pH 6.0.

In conclusion, fungal lipases are one of the enzymes having huge market demand. Optimization studies on media parameters for maximum lipase activity were done on selected lipolytic fungi. Total three lipolytic fungi have shown a broad range of pH and temperature. For a maximum lipase production, carbon and nitrogen sources are also important.

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References

Atlas, R. M., (1993). **Handbook of Microbiological Media.** CRC Press, London.

Alexander, S. K. and D. Strete, (2001). **Microbiology: A Photographic Atlas for The Laboratory.** U.S.A.

Barnett, H. L., (1960). **Illustrated Genera of Imperfected Fungi.** Second Edition. Printed in the United States of America.

Basheer, S. M., (2007). **Lipase production by marine fungus aspergillus awamori nagazawa.** Kerala, India.

Brooks A. A. and N. U. Asamudo, (2011). **Lipase production by strains of Aspergillus species isolated from contaminated body creams.** Nigeria. Journal of Toxicology and Environmental Health Sciences Vol. 3(11), pp. 311-316.

Bakir, Z. B and K. Metin, (2017). **Production and characterization of an Alkaline lipase from Thermophilic *Anoxybacillus* sp. HBB16.** Turkey. Biochem. Eng. Q., 31 (3) 303-312.

Colla, L. M., A. M. M. Ficanha, M. J. Rizzardi, T. E. Bertolin, C. O. Reinehr and J. A. V. Costa, (2015). **Production and characterization of lipase by two new isolates of Aspergillus through solid-state and submerged fermentation.** BioMed Research International, ID 725959.

Dube, H. C., (1983). **An Introduction to Fungi. Second Revised Edition.** Printed at Vishal Printers, Delhi.

Griebeler, N., A. E. Polloni., D. Remonatto, F. Arbter., R. Vardanega, J. L. Cechet, M. D. Luccio, D. d. Oliveira, H. Treichel, R. L. Cansian, E. Rigo and J. L. Ninow, 2011. **Isolation and screening of lipase-producing fungi with hydrolytic activity.** Food Bioprocess Technol 4: p. 57-586.

Kanwar, S. S., R. K. Kaushal, A. Jawed, R. Gupta and S. S. Chimni, (2005). **Methods for inhibition of residual lipase activity in colorimetric assay: A comparative study.** Indian Journal of Biochemistry and Biophysics, Vol. 42, pp. 233-237.

Kotogan, A., B. Nemeth, C. Vagvolgyi, T. Papp and M. Tako, (2014). **Screening for extracellular lipase enzymes with transesterification capacity in Mucoromycotina strains.** ISSN (FTB-3455), p. 1330-9862.

Lanka. S and T. Trinkle, (2017). **Screening and Isolation of Lipase Producing Fungi from Marine Water Obtained from Machilipatnam Costal Region.** International Journal of Pharmacognosy and Phytochemical Research; 9 (7); 928 - 932, India.

Massadeh, M. I., and Sabra, F. M., (2011). **Production and characterization of lipase from *Bacillus sterarothermophillus*.** Jordan. African Journal of Biotechnology, Vol. 10(61), pp. 13139-13146.

Niaz, M., T. Iftikhar, F.F. Qureshi and M. Niaz, (2014). **Extracellular lipase production by *Aspergillus* nidulans (MBL-S-6) under submerged fermentation**. Pakistan. Int. J. Agric. Biol., 16: 536_542.

Prabhakar, T. K. A. Kumar and P. Ellaiah, (2002). **The effect of cultural conditions on the production of lipase by fungi.** Journal of Scientific and Industrial Research, Visakhapatnam. Vol. 61, pp- 123-127.

Pandey. H., A. Kestwal, D. Chauhan, S. Kumari, V. Dhalwal, G. J. Singh, P. Singh, Parul Mann, A. Sharma, G. Saxena, A. Kapoor and B. Giri, (2016). **Isolation and screening of potential fungi and standardization of a process for the production of exracellular lipase.** DU Haurnal of undergraduatae Research and Innocation (Delhi, 2016): p. 116-122.

Rodrigues. C., S. T. A. Cassini, P. W. P. Antunes, L. M. Pinotti, R. P. Keller and R. F. Goncalves, (2016)**. Lipase producing fungi for potential wastewater treatment and bioenergy production.** Academic journal of biotechnology. Vol. 15(18), pp. 759-767. Africa.

Sirisha, E., N. Rajasekar and M. L. Narasu, (2010). **Isolation and optimization of lipase producing Bacteria from Oil contaminated soils.** India. Advances in Biological Research 4 (5): 249-252.

Shukla, B. N and P. V. Desai, (2016). **Isolation, characterization and optimization of lipase producing *Pseudomonas* spp. from oil contaminated sites.** India. Int.J.Curr.Microbial.App.Sic. 5(5): 902-909.

Winkler, U. K and M. Stuckmann, (1979). **Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by Serratia marcescens.** Journal of Bacteriology, 138, 663-670.

Wadia, T. and S. K. Jain, (2017). **Isolation, screening and identification of lipase producing fungi from oil contaminated soil of Shani Mandir Ujjain.** Int. J. Curr. Microbiol. App. Sci (2017) 6(7): p. 1872-1878.

Websites

www.Shodhganga.inflibnet. ac>bitstream.com