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Research Article

# ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF LIPASE PRODUCING MICROORGANISMS FROM ENVIRONMENT

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**ABSTRACT**

**Objective:** Lipases represent an important group of hydrolytic proteins. These enzymes find numerous applications in the industrial sector to provide regioselective molecules of commercial interest. The present investigation was carried out to isolate and identify a bacterium that can quickly produce this enzyme from very simple and cost effective nutrient medium.

**Materials and Methods:** Lipid as a sole source of carbon as a selection pressure in the nutrient medium was applied. Isolate was identified, and enzyme activity was quantitatively assayed. Lipase production was characterized.

**Results:** The manuscript provides information about the isolation and identification of *Enterobacter* spp.

**Conclusion:** The isolate produces enzyme very early in its logarithm phase of life cycle and can be a promising candidate of lipase.

**Keywords:** Lipase, Isolation, Para nitrophenol palmitate.

## INTRODUCTION

Lipase (Triacylglycerol hydrolases EC 3.1.1.3) is an enzyme capable of hydrolyzing lipids into fatty acids and glycerol [1]. It possesses many industrial applications such as pharmaceuticals, food, detergents, paper and pulp, agrochemicals, biosurfactants and bioremediation, etc. [2,3]. Microbial lipases have already established their vast potential regarding usage in numerous applications. Specifically, they are employed in waste water treatment (degreasing of lipid clogged drains), pharmaceutical (resolution of racemic mixtures), dairy (hydrolysis of milk, fat), leather (removal of lipids from hides and skin), detergent (removal of oil/fat stains) and medical (diagnostic tool in blood triglyceride assay) industries [4]. The major commercial application for hydrolytic lipases is their use in laundry detergents. Detergent enzymes makeup nearly 32% of the total lipase sales. Lipase for use in detergents needs to be thermostable and remain active in the alkaline environment of a typical machine wash. An estimated 1000 tons of lipases are added to approximately 13 billion tons of detergents produced each year [5].

Lipases can play an important role in the processing of g-linolenic acid, a polyunsaturated fatty acid; astaxanthin, a food colorant; methyl ketones, flavor molecules characteristic of flavor molecules characteristic of blue cheese [6]; 4-hydroxydecanoic acid used as a precursor of g-decalactone, a fruit flavor; dicarboxylic acids for use as prepolymers; interesterification of cheaper glycerides to more valuable forms (e.g., cocoa butter replacements for use in cchocolate manufacture) [7]; modification of vegetable oils at position 2 of the triglyceride, to obtain fats similar to human milk fat for use in baby feeds; lipid esters, including isopropylmyristate, for use in cosmetics; and monoglycerides for use as emulsifiers in food and pharmaceutical applications.

The increasing awareness of the importance of chirality in the context of biological activity has stimulated a growing demand for efficient methods for the industrial synthesis of pure enantiomers, including chiral antiinflammatory drugs [8] and antihypertensive agents such as angiotensin-converting enzyme inhibitors (e.g., captopril, enalapril, ceranopril, zofenapril, and lisinopril); and the calcium channel blocking drugs. Lipases are used in the synthesis of these drugs [9].

Lipases can be produced from various sources, e.g., animals, plants and microorganisms. However, for industrial applications, lipases from microorganisms are more interesting because (1) they can be produced in the high yields (2) there are many varieties of catalytic activities that can be used in many applications. (3) The genetic manipulation is easily available [10].

Lipase producing bacterial strains are generally widespread in nature [11]. Lipase producers have been isolated mainly from soil, or spoiled food material that contains vegetable oil. In view of the above applications of lipase in numerous commercial processes, the present study was carried out with an objective to isolate and characterize lipase producing microorganisms from environmental samples that can produce lipase in a very shorter time period after inoculation in the production medium.

## MATERIALS AND METHODS

### Isolation of lipolytic microorganisms

The isolation was done by following the procedures of [12]. Sample from various locations (oily sewage water; oily water; detergent water; oily soil; diesel soil; ghee; sweet shop waste; garden soil; sewage pond water [SPW]) were collected and spreaded on defined medium (Table 1). All incubations for isolation were done at 37°C for 48 hrs

**Table 1: Composition of isolation medium: Tributyrin agar**

| Components | Quantity |
|---|---|
| Peptone | 0.5% |
| Yeast extract | 0.3% |
| Ammonium nitrate | 1% |
| $K_2HPO_4$ | 0.05% |
| $KH_2PO_4$ | 0.05% |
| Glucose | 0.1% |
| Tributyrin/Mustard oil | 1% |
| Agar | 2% |
| pH | 8 |

along with control. The colonies appeared were marked according to their distinct morphological appearances. It was followed by screening by qualitative and quantitative determination of enzyme activity.

**Quantitative/volumetric estimation of enzyme activity**

The above obtained bacterial growth was purified, and isolated colonies were grown into tributyrin broths at 37°C with a rpm of 150 along with a control for 24 hrs. The bacterial growth harvested at 10,000 rpm for 5 minutes at room temperature to obtain supernatant and resting cells. The resting cells were studied for their morphological features by Gram's staining, while the supernatant was used as a source of extracellular enzyme to check its activity quantitatively. Lipase activity was determined spectrophotometrically by measuring the amount p-nitro phenol produced from the enzymatic hydrolysis of Lipid using the p-nitrophenol palmitate as a substrate by following the methodology of Winckler and Stuckmann [13] with some modifications.

The amount of p-nitro phenol formed was determined with the help of a standard curve (1-10 µg/mL). One unit of lipase activity was defined as the amount (mL) of the enzyme required to produce 1 nmol of pNP per minute under assay conditions. The isolate with highest lipase activity was selected and further subjected to the identification process.

**Morphological and biochemical characterization**

The selected isolates were examined for morphology, biochemical characteristics and enzymatic activities (Catalase test, $H_2S$ production, MR-VP Test, Indole production and carbohydrate fermentation test). A set of nine different sugars was used for this test namely, glucose, galactose, fructose, mannose, xylose, sucrose, mannitol, glycerol, and lactose.

**Growth curve preparation**

The growth curve was prepared in a triplicate manner in nutrient broth and tributyrin broth media. A loop full of the bacterial culture was inoculated into 100 mL of nutrient broth and incubated at 30°C with a rpm of 150 along with a control. Absorbance values were noted down at 660 nm, until 27 hrs with an interval of every 3 hrs.

**Time of enzyme production**

The time of enzyme production in tributyrin broth was determined by inoculating 5% inocula of nutrient broth of the exponential phase into tributyrin broth tubes, again in triplicates and incubating at 30°C with a rpm of 150 along with a control. Amylase activity of the bacterial culture supernatant was determined as above from 3 to 33 hrs of growth.

## RESULTS AND DISCUSSION

**Isolation of lipolytic microorganisms**

The inoculated environmental samples on tributyrin agar plates exhibited various types of microbial growth on incubation. The control petriplate showed no growth. The obtained growth was envisaged as follows (Table 2).

The absence of colonies in ghee, sweet shop waste soil and oily water can be attributed to the inability of the microorganisms to grow on tributyrin agar medium due to incapable of utilizing the lipid as a substrate. Lipase production from a variety of bacteria, fungi and actinomycetes has been reported in several works [14,15] most of them have been isolated mainly from soil, or spoiled food material that contains vegetable oil.

**Qualitative determination of amylase activity**

The ability to degrade lipid is used as a criterion for the determination of lipase production by a microbe depending upon the zone of hydrolysis produced. The marked isolates showed their response variably as zone of hydrolysis as given in Table 3. Similar results were obtained by Mobarak-Qamsari *et al.* [12] and Prasanna et *al. [1*6].

Positive results mean that the isolate has shown the zone of hydrolysis around their colony, while the negative results means absence of a clear zone around the microbial colonies. The absence of a clear zone may be due to intracellular enzyme production. It was further confirmed by quantitative estimation of enzyme activity.

**Quantitative estimation of lipase activity**

The isolates were screened on the basis of quantitative estimation of enzyme activity. The isolates showed following enzyme activity values (Table 4).

It is evident from the above table that the "SPW" has maximum extracellular lipase activity. Thus, it was selected for further experiments.

**Identification of the isolate**

The morphological characterization revealed following results (Table 5).

**Growth curve of the isolate**

A growth profile was obtained by sketching a curve between optical density readings at 660 nm and the time intervals in nutrient broth and tributyrin broth media. The isolate shows maximum growth after 15 hrs of inoculation into nutrient broth medium while it grows maximally after 9 hrs of inoculation in tributyrin broth medium.

In nutrient broth, the log phase starts after 3 hrs of inoculation and after 15 hrs of inoculation the bacteria reach into its stationary phase of growth (Fig. 1). In tributyrin broth, the log phase comes after 2 hrs of inoculation and stationary phase after 15 hrs of incubation (Fig. 2).

**Table 2: Characterization of microbial growth on tributyrin agar isolation medium**

| Sample | Growth | Isolate |
|---|---|---|
| Oily sewage water | Gram-positive, bacilli | OSW |
| Detergent water | Gram-positive, bacilli | DW |
| Diesel soil | Gram-negative, bacilli+ cocci shaped (mixed culture) Gram-positive, bacilli+ cocci shaped (mixed culture) | DS |
| Oily soil | Gram-negative, bacilli+ cocci shaped (mixed culture) | OS |
| Sewage pond water | Gram-negative, bacilli | SPW |
| Garden soil | Gram-positive, bacilli | GS |

OSW: Oily sewage water, DW: Detergent water, DS: Diesel soil, OS: Oily soil, SPW: Sewage pond water, GS: Garden soil

**Table 3: Qualitative analysis of amylase production by the isolates**

| Isolates | Zone of hydrolysis presence |
|---|---|
| OSW | Negative |
| DW | Negative |
| DS | Negative |
| OS | Negative |
| SPW | Positive |
| GS | Positive |

OSW: Oily sewage water, DW: Detergent water, DS: Diesel soil, OS: Oily soil, SPW: Sewage pond water, GS: Garden soil

**Table 4: Volumetric enzyme activity of isolate**

| Isolates | Volume activity (nmol/ml/min) | |
|---|---|---|
| | Resting cell suspension | Supernatant |
| OSW | 7.19±0.07 | 0.0 |
| DW | 5.75±0.05 | 0.0 |
| SPW | 0.0 | 0.791±0.07 |
| GS | 1.43±0.01 | 0.28±0.02 |

OSW: Oily sewage water, DW: Detergent water, SPW: Sewage pond water, GS: Garden soil

**Table 5: Morphological and biochemical features of the isolate**

| Characteristics | Observation |
|---|---|
| Culture morphology | Yellow, smooth, mucoid colonies on nutrient agar plate |
| Shape | Gram-negative rods |
| Spore formation | Absent |
| Capsule | Absent |
| Catalase activity | Present |
| MR test | Present |
| VP test | Absent |
| $H_2S$ production | Absent |
| Indole production | Absent |
| **Carbohydrate utilization test (Acid formation and gas production)** | |
| Xylose | Acid without gas |
| Glycerol | Acid without gas |
| Galactose | Acid without gas |
| Mannitol | Acid without gas |
| Mannose | Acid without gas |
| Fructose | Acid without gas |
| Glucose | Acid without gas |
| Lactose | Acid without gas |
| Sucrose | No acid, no gas |

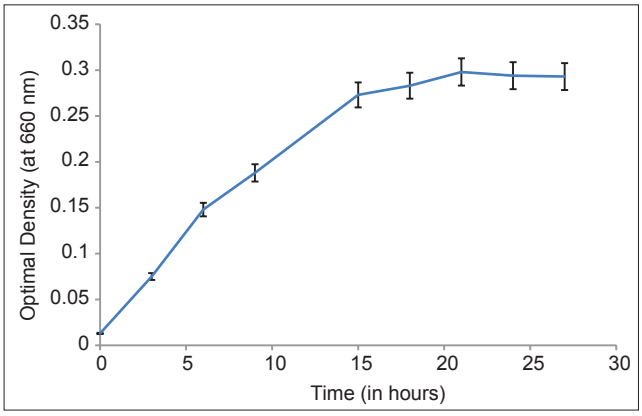


**Fig. 1: Growth curve of the isolate in nutrient broth**

In nutrient broth, the bacterial culture inoculated from tributyrin broth has shown a prolonged log phase. Growth is observed only after 3 hrs of incubation. This may be due to rapid acclimatization of the isolate in a new medium. The log phase in nutrient broth is shorter than the log phase in tributyrin broth. It can be assumed that the bacterium consumes the nutrients in the nutrient broth more rapidly than in starch broth.

**Phase of enzyme production**

The time at which selected isolate produces lipase enzyme was determined by determining the enzyme activity at various time intervals. Maximum production was observed after 6 hrs of inoculation (Fig. 3).

The highest activity was obtained at 6 hrs of growth and was consistent with the log phase of the bacterium.

## CONCLUSION

The present study was aimed to isolate and characterize lipase producing microorganism from various samples. Four bacterial isolates were selected on the basis of their growth on medium containing lipid as a sole source of carbon and zone of hydrolysis. On screening by quantitative estimation, an isolate found to be the highest lipase producer in supernatant was selected, and further studied. The strain was characterized biochemically by employing "Bergey's Manual of Determinative Bacteriology" and was identified as *Enterobacter* spp. The growth curves were prepared in nutrient and tributyrin broth

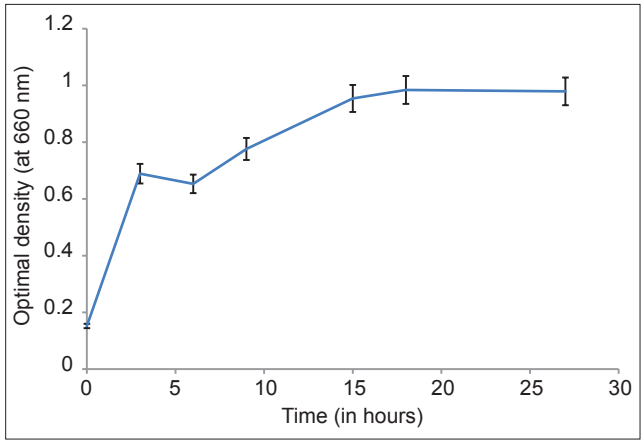


**Fig. 2: Growth curve of the isolate in tributyrin broth**

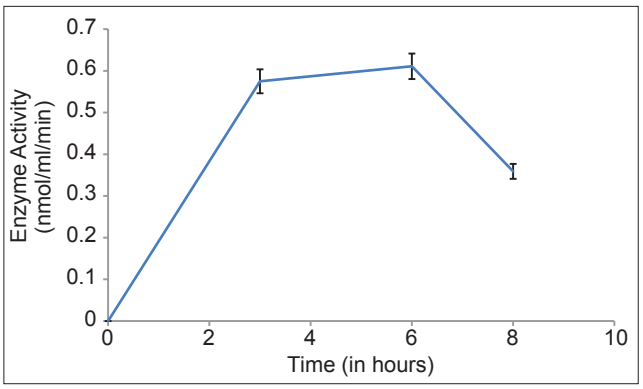


**Fig. 3: Enzyme activity at various time periods in tributyrin broth medium**

media and phase of enzyme production was determined. Initially, the isolate appeared promising and can be a good source of lipase but it requires a detail characterization of growth and nutrient conditions.

## REFERENCES

1. Yasuo S, Masahito H, Koichi H. Use of tween 20 as a substrate for assay of lipase activity in soils. Soil Sci Plant Nutr 2002;48(5):729-34.
2. Pogori N, Xu Y, Cheikhyoussef A. Potential aspects of lipases obtained from *Rhizopus* fungi. Res J Microbiol 2007;2(2):101-16.
3. Sharma DB, Shukla AK. Biotechnological approach of microbial lipase - A review. Biotechnology 2011;10:23-40.
4. Kumar S, Kikon K, Upadhyay A, Kanwar SS, Gupta R. Production, purification, and characterization of lipase from thermophilic and alkaliphilic Bacillus coagulans BTS-3. Protein Expr Purif 2005;41(1):38-44.
5. Reetz MT, Jaeger KE. Overexpression, immobilization and biotechnological application of *Pseudomonas lipases*. Chem Phys Lipids 1998;93(1-2):3-14.
6. Begam MS, Pradeep FS, Pradeep BV. Production, Purification, Characterization and Applications of Lipase from Serratia Marcescens MBB05. Asian Journal of Pharmaceutical and Clinical Research 2012;5(4):235-47.
7. Undurraga D, Markovits A, Erazo S. Cocoa Butter Equivalent Through Enzymic Interesterification of Palm Oil Midfraction. Process Biochem 2001;36:933-9.
8. Xin JY, Li SB, Xu Y, Chui JR, Xia CG. Dynamic Enzymatic Resolution of Naproxen Methyl Ester in a Membrane Bioreactor. J Chem Technol Biotechnol 2001;76:79-85.
9. Kazlauskas RJ, Bornscheuer UT. Biotransformations with lipases. In: Rehm HJ, Pihler G, Stadler A, Kelly PJW. Biotechnology. New York, VCH. 1998;8:37-192.
10. Hasan F, Shah AA, Hameed A. Industrial applications of microbial lipases. Enz Microb Technol 2006;39:235-51.
11. Veerapagu M, Narayanan AS, Ponmurugan K, Jeya KR. Screening

Selection Identification Production and Optimization of bacterial lipase from oil spilled soil. Asian Journal of Pharmaceutical and Clinical Research 2013;6(3):62-7.

12. Mobarak-Qamsari E, Kasra-Kermanshahi R, Moosavi-nejad Z. Isolation and Identification of a Novel, Lipase-Producing Bacterium, Pseudomnas aeruginosa KM110. Iran J Microbiol 2011;3:92-8.
13. Winckler UK, Stuckmann M. Glycogen, Hyaluronate, and some other Polysaccharide greatly Enhance the Formation of Exolipase by Serratia marcescen. J Bacteriol 1979;138:663-70.
14. Sztajer H, Maliszewska I. The Effect of Culture Conditions on Lipolytic Productivity of Penicillium citrinum. Biotechnol Lett 1989;11:895-8.
15. Kulkarni N, Gadre RV. Production and properties of alkaline, thermophilic lipase from Pseudomonas fluorenscens NS2W. Journal of Industrial Microbiology and Biotechnology 2002;28:344-8.
16. Prasanna RM, Mahendran VS, Balakrishnan V. Isolation and Identification of Lipase Producing Organisms from Diverse Soil Samples of Kolli Hills. International Journal of Current Microbiology and Applied Science 2013;1:205-10.