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DESTAINING POTENTIAL OF BACTERIAL LIPASE ENZYME ISOLATED FROM *PROVIDENCIA RETTGERI* INHABITING THE FRESH WATER FISH *MYSTUS BLEEKERI*

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ABSTRACT

The objectives of present study were to isolate and identify lipase producing bacterial strain *Providencia rettgeri* (MB3) was isolated from fresh water fish *Mystus bleekeri.* Total of 6 bacterial strains were obtained in the fish tissues. Screenings of lipase production were tested on tributyrin agar plate. Among the 6 isolates, MB3 was showed maximum zone of hydrolysis around the well. Partial purification of crude enzyme produced from *P. rettgeri* (MB3) was carried out by ammonium sulfate precipitation and dialysis further purified for DEAE-Cellulose column chromatography. The optimization of lipase enzyme was active at p^H 8, temperature 40°C, incubation 48 hrs, carbon source as lactose and best nitrogen source was peptone. According to our knowledge, this study demonstrated the first report on alkaline lipase producing to *P. rettgeri* (MB3) isolated from fresh water fish. The partially purified lipase enzyme was effectively removed by the blood stains and paves the way for its possible utilization in textile industry in the near future.

KEYWORDS: Lipase enzyme, *Providencia rettgeri*, *Mystus bleekeri*, Optimization, Blood stain.

1.0 INTRODUCTION

Enzymes are biological catalyst, highly specialized with extraordinary catalytic power comprise remarkable specificity. Lipase is an ester hydrolase, which catalyzes the hydrolysis

of long-chain triacylglycerols to form glycerol and fatty acid in the presence of excess water (Pascale *et al*., 2008). Microbial lipases are one of the most important extracellular enzymes which have been the mainly focused on scientific researchers due to its enormous biotechnological usage over years (Ullah *et al*., 2015).

Microbes favored lipase enzymes for fermentation bioprocesses due to their fast growth rate genetically engineered to produce new enzyme with necessary abilities. Lipase has been applied in the modern food industry instead of traditional chemical processes and used in the production of a variety of products like fruit juices, baked foods, fermented vegetables, cheese, soups sauces, pharmaceuticals, paper manufacturing, production of cosmetics and environmental bioremediations (Massadeh, 2012; Mehta, 2016). This enzyme is found throughout all kingdoms of life, which are prokaryotes including bacteria and archaea and eukaryotes including plants, animals and fungi (Cai-hong *et al*., 2008).

Microbial lipases are more stable than their corresponding plant and animal origin and their production is more convenient, safer and can be obtained in bulk at low cost (Vakhlu and Kour, 2006). Fishes obtain bacteria in the digestive tract from the aquatic environment through water and food that are populated with bacteria being rich in nutrient, the environment of digestive tract of fish confers a favorable culture environment for the microorganisms (Dehler *et al*., 2016; Pingle *et al*., 2016). In order to increase the cell yields and the enzymatic activities of the cells or to produce altered enzymes, genetic and environmental manipulations can be performed more readily on bacterial cells due to their short generation times, their simple nutritional needs and easy screening procedures for desired properties (Hasan *et al*., 2006).

The most important among the lipase producing bacteria are *Bacillus sp.* and variety of lipases are produced from both Gram positive and Gram negative bacteria, but greater part of the bacterial lipases comes from Gram-negative bacteria (Rasmey *et al*., 2017). The production of lipases by a microbial cell depends on the presence of a lipid, such as olive oil or any other inducer, such as triacylglycerols, fatty acids and tweens in the culture medium (Treichel *et al*., 2010). Olive oil is considered the most suitable lipid substrate due to its advantage of including high concentrations of oleic acid and being more economical (Jensen, 1983).

The effects of some variables on the activity of crude and purified lipases have been studied by some researchers. The different factors such as p^H, temperature, metallic ions, organic solvents, among others, can increase or decrease lipase activity. Extracellular enzyme can increase or decrease lipase production in microorganisms is highly influenced by medium component such as carbon sources, nitrogen sources and physical factors such as aeration, inoculum, size, p^H, temperature and incubation time (Smaniotto *et al*., 2014).

The main objective of the present work was to investigate the lipase production, in the fish bacterial isolate. In general, no defined medium has been established for the best production of any metabolite because the genetic diversity present in the microbial sources causes each organisms or strain to possess exclusive nature for maximum production. Therefore, it is essential to have a detailed investigation on newly isolated bacterial strain for production pattern under different physical parameter and cultural conditions were also optimized in the present investigation.

2.0 MATERIALS AND METHODS

Collection, isolation and screening of lipase producing strain.

The fresh water fish *Mystus bleekeri* was collected from Chambarambakkam lake (Latitude 13.0114^o N and Longitude 80.0591^o E), near Chennai, Tamil Nadu, India [Plate-1]. The external surface of the fish was thoroughly washed with running tap water and then it is washed with 70% ethanol to remove the external debris. The fish were dissected on laminar chamber and dissected out gill, skin and gut tissue of the selected fish was aseptically removed [Plate-2]. One gram of each tissue was pounded with mortar and pestle according to the method of Shinkafi and Ukwaja (2010). Each tissue samples were serially diluted and 100 µl of sample were plated on solidified agar medium with the help of L- rod it was spread uniformly onto the medium. The plates were incubated at 37^{o}C for 24 hours and examined for the development of bacterial colonies [Ibrahim *et al*., 2014].

Screening of lipase enzyme (Tributyrin agar plate assay)

Screening of lipase enzyme was ascertained according to the method of Padmapriya *et al*. (2011). Preliminary screenings of lipase to form a well of 6mm diameter in tributyrin agar plate was punched aseptically with sterile cork borer. Then crude lipase enzyme of 100µl was loaded in each well and kept for incubation at 37^{o}C for 24 hours and observed for zone formation. A clear zone around the well indicates the production of lipase [Plate-3].

Quantitative analysis of lipase enzyme

Titrimetric method as described by (ACS Specification, 1993) was adapted to determine the lipase activity on basis of olive oil hydrolysis. One ml of the culture supernatant was added to the reaction mixture containing 1ml of 200mM Tris-HCl buffer (p^H7.7), 2.5ml of deionized water and 3ml of olive oil. The solution was mixed well and kept 37^{o}C for 30 minutes. Both test and blank were prepared. After 30 minutes the test solution was transferred to a 50ml of Erlenmeyer flask. The reaction is terminated by adding 3ml of 95% ethanol liberated fatty acid was titrated against 50mM NaOH using Thymolphthalin as an indicator. Appearance of blue colour was considered as an end point. A unit of lipase defined as the amount of enzyme which released one micromole fatty acid from olive oil/min per ml under standard assay condition [Plate-4].

The enzyme activity was calculated from the formula

$$\text{Units/mL Enzyme} = \frac{\text{Vol. of NaOH consumed (mL) X Molarity of NaOH}}{\text{Volume of enzyme assay used X length of assay X volume used in Spectrophotometer determination}}$$

$$\text{Specific activity (U/mg)} = \frac{\text{Total lipase activity}}{\text{Total protein activity}}$$

Protein estimation by Lowry's method

Protein concentration of soluble enzyme preparation was quantified by method of Lowry *et al*. (1951) using Bovine Serum Albumin (BSA) as a standard.

Optimization of culture conditions for enhanced lipase enzyme production

Potential bacterial isolate *P. rettgeri* was selected for optimization study using standard method was adopted to optimize the parameters like pH, temperature, incubation time carbon source and nitrogen sources followed method by Bakir and Metin (2015) ; Rajanikanth and Damodharam (2016).

Partial purification of lipase enzyme

Bacterial culture broth of *P. rettgeri* was centrifuged for 10 min at 10,000 rpm in order to obtain culture supernatant which contains crude lipase enzymes.

Ammonium sulfate precipitation and desalting via dialysis

For ammonium sulfate precipitation solid ammonium sulfate was added to supernatant at 40% - 80% level saturation and precipitation was centrifuged. Protein solution was put into a dialysis tube tightened at the ends and it was placed in a large amount of de-ionized water

and stirred for 16 to 24 hours. After repetition of equilibrium process several times the level of salt concentration was reached to minimum level. This dialyzed sample was assayed and subjected to DEAE cellulose column chromatography (Rajeswari *et al*., 2011; Fatima *et al*., 2014; Muthazhagan and Thangaraj, 2014) & [Plate-5].

Diethylaminoethyl cellulose (DEAE) Column chromatography

The dialyzed solution containing both soluble and insoluble material was put together on cellulose powder column (2.5×15cm) equilibrated with 10mM phosphate buffer, p^H 7.5. The column was washed with four bed volumes of the same buffer. The sample was eluted with a linear NaCl gradient (2 L), from 0 to 0.8M, in the same buffer. The flow rate was adjusted to 1mL/min (fraction volume: 5mL). The lipase assay and protein estimations were performed to each fraction. The lipase rich fractions were pooled and stored at 4°C.

Removal of blood stain from cloth using partially purified lipase enzyme

Application of lipase removal of blood staining was studied on white cotton cloth pieces (4×4 cm) stained with blood stain. The removal blood stain was observed in various time periods such as 20, 30 & 40 minutes. Visual examination of various cloth pieces exhibited the effect of partially purified lipase enzyme in removal of stains. Blood stained cloth pieces with distilled water were taken as control (Mukeshkumar et al., 2012).

3.0 RESULTS AND DISSCUSSION

Isolation and screening of lipase producing bacteria

The total of six bacterial strains MB1to MB6 was obtained from fresh water fish. The potent enzyme producer was identified *P. rettgeri* (MB3). Among the six isolates, especially MB3 was observed in maximum zone around well in 22mm [Table-2]. Lipase producing bacterial isolate *P. rettgeri* was exhibited best lipolytic ability by hydrolysis for tributrin agar plate [Plate-3]. Similar studies were reported that Dutta and Ghosh (2015) extracellular amylase, protease, lipase, cellulase, phytase and xylase enzymes producing strains were isolated from gastrointestinal tract of *Cirrhinus mrigala.* According to Jini *et al*. (2011) were studied proteolytic and lipolytic lactic acid bacteria were isolated from visceral wastes of different freshwater fishes *Labeo rohita*; *Cirrhinus mrigala*; *Catla catla*; *Cyprinus carpio* and *Oreochromis mossambicus*. Kamble (2017) was reported *Exiguobacterium* sp (SK1 and SK2) the excellent qualitative lipase activity was found in with similar zone of hydrolysis was 30mm.

Effect of p^H on lipase production

The enzyme activity of lipase enzyme with *P. rettgeri* at p^H 4,5,6,7,8 & 9 were observed as 111 (U/ml), 132 (U/ml), 151(U/ml), 169 (U/ml), 180 (U/ml) and 121(U/ml) respectively. The optimum p^H for maximum production of protease was p^H-8.0 [Figure-1]. The similar optimization studies were reported that Padmapriya *et al*. (2011); Veerapagu *et al*. (2013); Fatima *et al*. (2014).

Effect of Temperature on production of lipase

The growth and enzyme activity by the bacteria are influenced by different incubation temperature. Enzyme activity of *P. rettgeri* at different temperature 20^{o}C, 30^{o}C, 40^{o}C, 50^{o}C and 60^{o}C were observed as 102.3 (U/ml), 134.5 (U/ml), 191.45 (U/ml), 138.9 (U/ml) and 109.7 (U/ml), respectively. The optimum temperature for maximum production of lipase was found to be 40^o C [Figure-2].

Effect of incubation period on production of lipase

Alkaline lipase production was tested on different incubation period such as 24, 48 and 72 hrs. It has been found to be maximum enzyme activity at 48 hrs. Incubation and the enzyme activity were analyzed 104.3 (U/ml), 205.42 (U/ml), and 143.72 (U/ml) respectively. The optimum incubation time was 48hrs [Figure-3].

Effect of carbon and nitrogen sources on production of lipase

Effect of carbon sources on production of lipase like glucose, maltose, lactose, starch and sucrose were tested and production of alkaline lipase activities were found to be 107.73 (U/ml), 154.26 (U/ml), 210.7 (U/ml), 170.5 (U/ml) and 105.8 (U/ml) respectively. Among five carbon sources lactose was found to be the best carbon source that increased the production of lipase by *P. rettgeri* (MB3), when compared to other carbon sources [Figure-4]. Similarly organic and inorganic nitrogen sources were tested on lipase such as ammonium nitrate (143.6 U/ml), ammonium sulphate (154.5 U/ml), potassium nitrate (165.9 U/ml), peptone (245.8 U/ml) beef extract (175.4 U/ml) and yeast extract (169.7 U/ml) of among the tested nitrogen sources, greatest lipase production was recorded with peptone at 245.8 (U/ml) as shown in the [Figure-5].

Table 1: Partial purification of lipase enzyme obtained from *P.rettgeri.*

Purification of lipase	Total lipase activity (U/ml)	Total Protein (mg/ml)	Lipase specific activity (U/mg)	Fold purification	Recovery yield (%)
Crude lipase enzyme	1052	7.08	148.58	1	100
$(NH_4)_2SO_4$ Precipitation (40-80%)	989	5.92	167.06	1.12	94
Dialysis	795	3.03	262.37	1.76	75
DEAE cellulose	688	1.51	455.62	3.06	65

The extracellular alkaline lipase enzyme produced by the *P. rettgeri* was partially purified by ammonium sulfate precipitation (40-80%) followed by dialysis and loaded on DEAE-cellulose chromatography. The lipase activity was analyzed for all the fractions obtained after partial purification. Among that a particular fraction which showed remarkable lipase activity was column eluted using gradient NaCl. The summary of lipase purification is presented in [Table-1]. The purified lipase specific activity was showed 455.62 U/mg. Overall, 65% recovery with 3.06 fold purification was achieved by three step purification process. Padmapriya *et al*. (2011) were made on lipase purification steps were also carried out to *Lactobacillus* spp. Mukeshkumar *et al*. (2012) has been reported lipase enzyme was purified with different ammonium sulfate precipitation and resultant dissolved in 20mM Tris HCl and dialysis against same buffer.

Removal of blood stained cloth using partially purified lipase enzyme

The removal blood stain was observed in various time periods such as 20, 30& 40 minutes. The test blood stained cloth piece soaked the lipase enzyme solution at different time durations. It has been effectively removal and observed after 40 minutes. It was visually observed [Plate-6A-D]. Mukesh kumar *et al*. (2012) has been proved that removing the blood stain stained cloth by *Serratia marcescens* crude enzyme with addition of detergent showed greater efficiency stain removal of blood.

In the present study the lipase producing bacteria were isolated from skin of *Mystus bleekeri*. Six different lipase producing strains were selected based on zone of hydrolysis. Among the six isolates the MB3 *P. rettgeri* exhibited maximum 22mm diameter of zone of hydrolysis in tributyrin agar medium.

Table 2: Screening of lipase enzyme (Tributyrin agar plate assay).

Tissue sample of *Mystus bleekeri*	Bacterial isolates	Tributyrin agar plate assay (mm)
Gill	MB1	15
	MB2	17
Skin	MB3	22
	MB4	20
Gut	MB5	14
	MB6	18

Plate-1: A photograph showing fresh water fish *Mystus bleekeri*

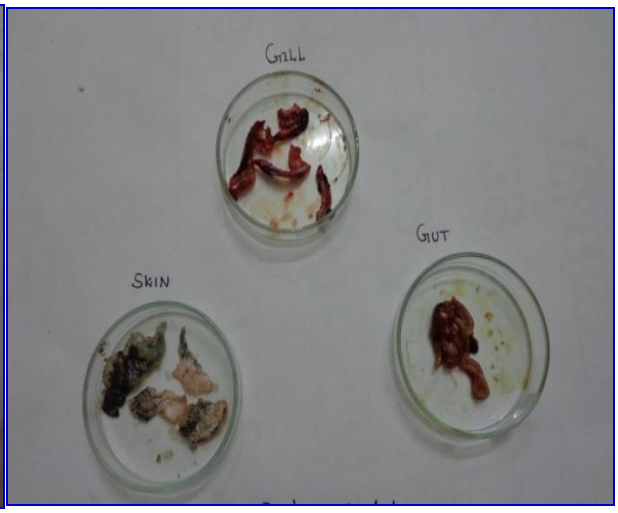


Plate-2: A photograph showing lipase producing bacterial srains obtained from gill, skin and gut tissues of *Mystus bleekeri*

Plate-3: A photograph showing qualitative analysis of lipase (Tributyrin agar plate)

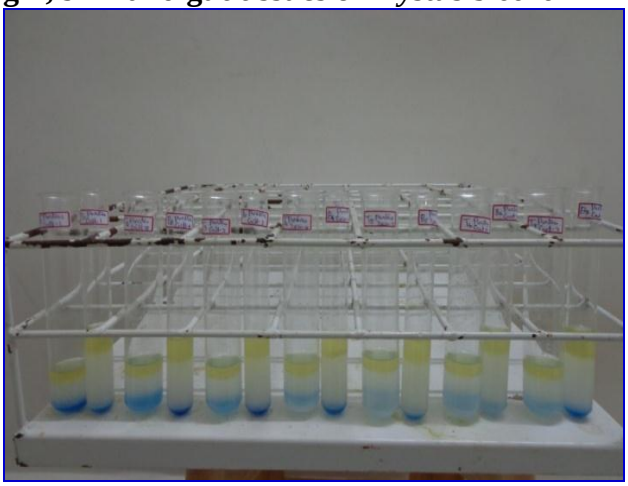
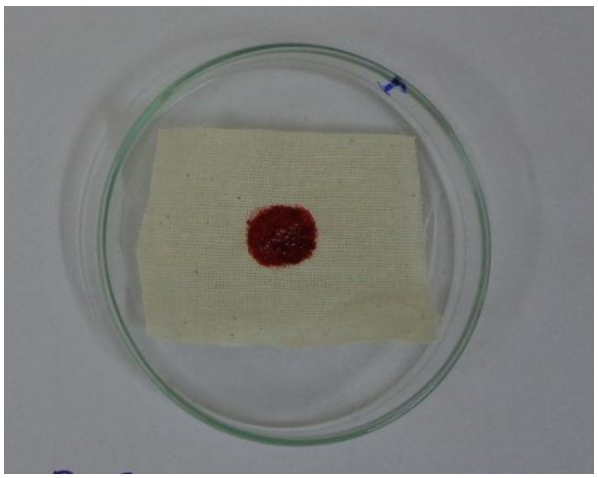
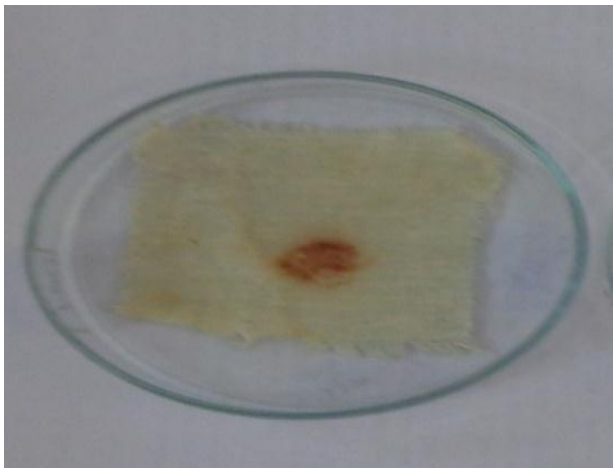


Plate-4: A photograph showing quantitative analysis of lipase enzyme using olive oil as substrate

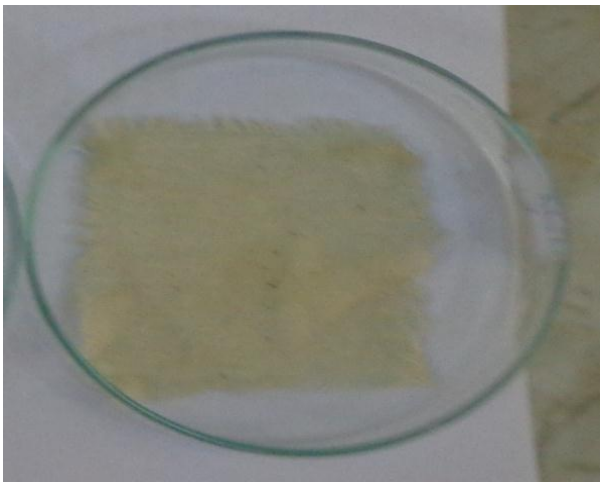
Plate-5: A photograph showing dialysis and partial purification of lipase enzyme



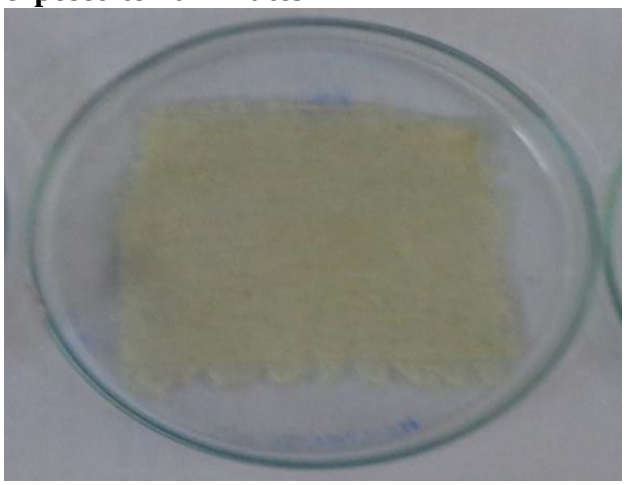
A. Destaining done with water (Control)



B. Destaining done with the lipase enzyme exposed to 20 minutes



C. Destaining done with the lipase enzyme exposed to 30 minutes



D. Destaining done with the lipase enzyme exposed to 40 minutes

Plate-6: A photograph showing removal of blood stain (destaining) from cloth piece using partially purified lipase enzyme.

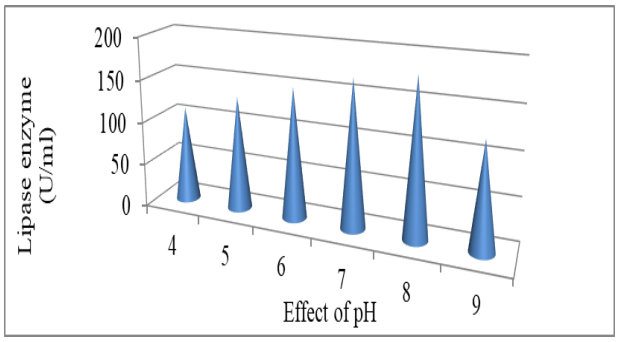


Figure -1: Effect of various pH on lipase production by *P.rettgeri*

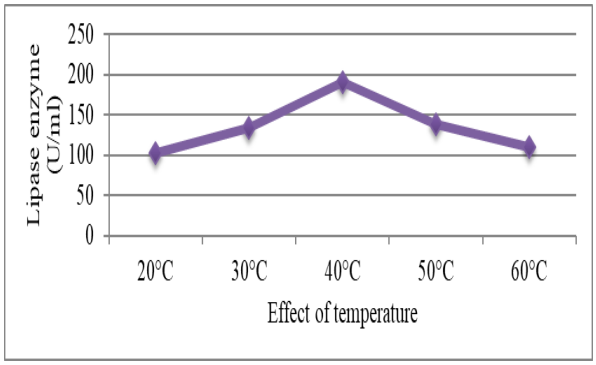


Figure-2: Effect of different temperature on lipase enzyme production by *P.rettgeri*

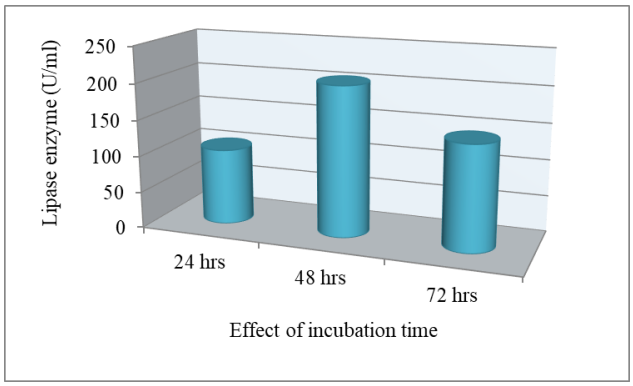


Figure-3: Effect of different incubation time on production of lipase enzyme by *P.rettgeri*

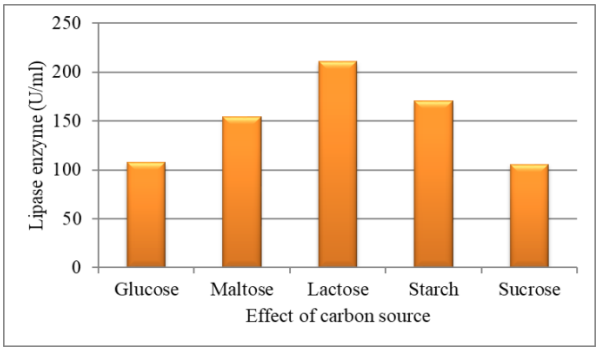


Figure-4: Effect of carbon sources on production of lipase by *P.rettgeri*

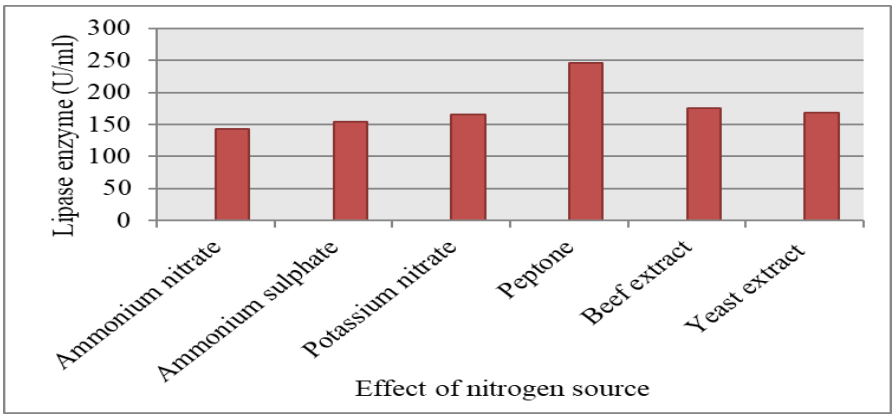


Figure-5: Effect of nitrogen sources on production of lipase by *P.rettgeri*

CONCLUSION

The work described the partial purification of lipase enzyme isolated from *P. rettgeri* (MB3) is gram negative bacteria and obtained from fresh water fish also forms the first report on the its lipase activity. Lipase enzyme could be used for large scale production of alkaline lipase enzyme needs in the industrial sector. In the present study lipase had an optimum p^H of around 8, the optimum temperature and incubation was 40^oC and 48hrs. The best carbon and nitrogen sources were lactose and peptone respectively. Therefore, the above said objectives

were achieved by reducing the cost, increasing the partially purified lipase enzyme which has tremendous applications in textile industry.

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