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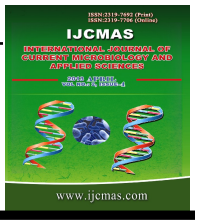

Original Research Article

Production and Optimization of Lipase from marine derived bacteria

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A B S T R A C T

The study proved that lipase producers are abundant in the sample collected from Vellar estuarine in Cuddalore district, Tamilnadu, South India which harboured a maximum density of 3.9×10^7 CFU/g. From the Tween 80 amended plates 5 strains were selected based on different morphology. They were purified and checked for their potential of lipase production using well assay using Tween 80 agar plate assay with Rodamine B. The strain with a zone of clearance was 16mm was selected as the most potential strain. The potential Strain SBS037 was selected as the most potential as it resulted in a zone of clearance was 16mm. The most potential strain was identified as *Pseudomonas putida* based biochemical tests, and it was designated as *Ps. Putida* SBS037. Culture conditions like temperature, pH and salinity, nutrients like carbon and nitrogen sources were found influence for growth. The identified optimized parameters were as follows: pH-7, temperature-35°C, Nacl-0.6%, dextrose-1% and yeast extract-0.2% and incubation time 48 hours. Mass scale cultivation with optimized parameter was done in shake flasks. The culture filtrate was precipitated with 60% ammonium sulphate and at the stage the 65.0 U/mL/min. of enzyme activity was found. After membrane dialysis activity increased to 85.0 U/mL/min. When cheaper source (i.e.) coconut oil cake at the rate of 10% was used instead of carbon and nitrogen sources surprisingly 147.0 U/mL/min. of enzyme was produced. SDS-PAGE analysis revealed that the protein profile of partially purified enzyme showed 34, 45 and 52 kDa proteins.

K e y w o r d s

Marine *Pseudomonas putida;* growth optimization; enzyme assay; SDS-PAGE.

Introduction

Enzymes are biocatalyst. Most are proteins (example: ribonucleoprotein). Enzymes are bind temporarily to one or more of the reactants of reaction and they lower the amount of activation energy needed and speed up the reaction. Lipase is a water soluble enzyme that catalyzes the hydrolysis of ester bonds in water insoluble, lipid substrates. Most of lipases act as the specific position on the glycerol backbone of lipid

substrates, which is the main enzyme to break down fats in human digestive system. It converts triglyceride to monoglycerides and free fatty acids.

Lipolytic enzymes are currently receiving enormous attention regarding their biotechnological potential (Benjamin and Pandev, 1998). They constitute the most important group of biocatalysts for biotechnological applications. In this application lipases for the synthesis of biopolymers and biodiesel, the production of enantiopure pharmaceuticals, agrochemicals and flavor compounds (Jaeger and Eggert, 2002).

The chemoregio and enantio-specific behavior of these enzymes caused tremendous interest among scientist and industrialists (Saxena *et al.,* 2003). Lipases are produced by animals, plants and microorganisms. Microbial lipases have gained special selectivity and broad substrate specificity (Dutra *et al*., 2008; Griebeler *et al*., 2009). Many microorganisms are known as potential producers of extracellular lipases including bacteria, fungi and yeast (Abada, 2008).

Genes encoding lipases are even present in certain viruses (Girod *et al*., 2002). Halotolerent and halophilic microorganisms grow in environments with high salinity concentrations. Halophiliic bacteria are found in different environments such as salts lakes, saline soils and salted foods (Ventosa *et al.,* 1998). Enzymes produced by halophilic microorganisms have developed particular features which confer them stability and solubility at high concentrations, thus low water concentrations.

Materials and Methods

Isolation and identification of lipase producing bacteria

For isolation of Lipase producing organisms, water and sediments were collected from the Vellar Estuary. 1.0g/1.0mL of sample was suspended in 99 mL sterile 50% aged sea water, agitated for 45 minutes in a shaker at 50^0C and 0.1 mL was spreaded on Tween 80 agar plates and incubated at 37^0C for 48 hrs. Strains with distinct zone of clearance were selectively isolated and the one with maximum activity was identified up to species level following Bergey's manual of Determinative bacteriology (Buchanan *et al.,* 1974) and the identified strain was stored in Tween 80 agar slant for further studies.

Determination of Lipase activity by titrimetric method

A very popular method to assay free fatty acids released by lipase action is the titrimetric method. In the fixed-time assays, the reaction is incubated for a period of time then stopped by the addition of a mixture of organic solvents, example: ethanol, acetone and ethyl ether. The released fatty acids are titrated against a dilute base solution like sodium hydroxide (NaOH) or potassium hydroxide (KOH) to an end point by using either an indicator like phenolphthalein or an automatic burette with a pH probe. The lipase activity is then expressed in units of activity and one unit is defined as one micro mole of fatty acid liberated in one minute.

A more reliable assay is the continuous titrimetric assay or the pH-stat, where the release of free fatty acids is continuously monitored throughout the incubation period and the pH of the reaction mixture is kept constant by the addition of a base solution

(Stauffer, 1989). The rate of addition of the base is a direct measure of the rate of free fatty acid formation. The reaction is usually carried out at a basic pH around 8.0-9.0. At pH below 8.0 less fatty acids are in their ionized form (Mattson and Volpenhein, 1966). This method is very valuable in the investigation of lipase kinetics, as the initial rate of the reaction can be determined directly.

Optimization of growth for potential strains

The identified potential strain was optimized for parameters like pH, temperature, salinity, carbon sources and nitrogen sources. The potential strain was subjected to different culture conditions to identify the optimum conditions for maximum enzyme production. Growth and lipase production were estimated at various temperatures (25, 30, 35 and 40ºC), pH (5, 6, 7, 8, 9 and 10) and salinity (Nacl-0.2, 0.4, 0.6, 0.8 and 1.0%).

Carbon sources like starch, cellulose, sucrose, maltose and dextrose were incorporated separately at the rate of 1.0% and their influence on growth was studied in 500 mL Erlenmeyer flasks. Likewise nitrogen sources like beef extract, peptone, yeast extract and ammonium nitrate were added at the rate of 0.2%. In all flasks 1.0% of Tween 80 was incorporated.

Growth was estimated at 600 nm in a UV spectrophotometer (Spectronics, India). The influencing parameters and nutrients were studied by selecting one parameter at a time at which other factors were kept as constant. Finally the optimized parameters were used to produce lipase enzyme in shake flask.

Mass scale culture of lipase producing bacterium

Based on the results obtained from the optimization such as pH 7.0, 35ºC, 0.6% Nacl, dextrose as carbon source and yeast extract as nitrogen source, the mass scale culture of the lipase producing organism was carried out. 1000 mL of production medium was inoculated with 10mL of inoculum containing 1.0 $\times 10^6$CFU/mL. The fermentation was carried out in 2000 mL Erlenmeyer flasks on a rotary shaker (300 rpm). At the end of the 48 hours the culture was harvested for the recovery of lipase enzyme from filtrate.

Mass scale culture of lipase producing bacterium using cheaper substrate

The potential strain was cultivated using coconut oil cake (10g/100mL).To the powdered coconut oil cake 10% was added in 1000 mL conical flask and kept the optimized culture conditions in a shaker at 150 rpm. Enzyme production was done as before.

Ammonium Sulphate precipitation and dialysis of the Lipase enzyme

The various steps of enzyme purification were carried out at 4ºC. In the initial purification step, the supernatant fluid containing the extra cellular enzyme was treated with solid ammonium sulphate as described by Green and Hughen, (1955) with continuous overnight stirring and separated in to the following saturation, ranging from 0-20%, 20-40%, 40-60% and 60-80%. The precipitate was collected by centrifugation in an ultra centrifuge at 3000 rpm for 30 minutes.

The precipitate was redissolved in Tris HCI (0.05 M, pH 8.0) buffer. The enzyme was dialyzed 3 times against the buffer. The enzyme activity was determined in both the precipitated and lyophilized proteins were assayed (Lowry *et al.,* 1951) under standard conditions.

Protein separation- SDS-PAGE (Laemmli, 1970)

The proteins were separated by SDS-PAGE electrophoresis and size of polypeptide chains of given protein can be determined by comparing its electrophoretic mobility in SDS-PAGE gel with mobility marker proteins of known molecular weight. Protein detection was done in coomassie brilliant blue staining.

Results and Discussion

In the present study, lipase producers were isolated from Vellar estuarine which harboured a maximum density of 3.9×10^7 CFU/g. About 5 strains were selected based on the measurement of zone of clearance. The potential strain SBS037 was selected based on the zone of clearance (16.0 mm). The most potential strain was identified as *Pseudomonas putida* based biochemical tests and designated as *Ps.putida* SBS037. Kanimozhi *et al.,* (2011) studied the lipase producing organism isolated from oil mill waste and identified it as *Bacillus subtilis.* Manoj *et al.,* (2010) found that the soil samples collected from oil refineries waste contaminated sites showed high bacterial count from 1.1×10^8 to 4.9×10^8 CFU/g. On the basis of larger clear zone formation on Tributylin agar, 8 potential isolates were selected in the study. The isolates were grown in tributylin broth at pH 8 and the supernatants were assayed for lipase activity after incubating for 48 hours. It was found that the isolate *Bacillus subtilis* was found to have highest enzyme production compared to other isolates.

In the present study *Pseudomonas putida* SBS037 was found to have maximum lipase activity at pH 7.0. In low and high initial medium pH tested, the lipase activity was found to be minimum. This result is in consistence with the earlier report of Achamma *et al.,* (2003). They inferred that the lipase activity of *Bacillus* spp. was maximum at pH 7.0. The study Ferrer *et al.* (2000) reported that the optimum pH ranges for lipase production was at pH 7 for *Penicillium chrysogenum.* Manoj *et al.,* (2010) clearly indicated that there is a strong influence of pH on lipase enzyme production. They reported the maximum activity at pH 8.0.

In the present study, the influence of temperature indicated that the lipase production by the isolated strains was higher at 35°C when compared to those at 25 and 40°C. Walavalkar and Bapat, (2001) reported the maximum lipase activity of *Staphylococcus* sp. at 37°C. Brockerhoff and Jensen, (1974) stated that lipases had a wide range of temperature for its activity (i.e.), 20ºC - 65ºC. According to Sheridan *et al.,* (2000) for the most of the enzyme the optimal temperature most enzymes have activity is around 30ºC-45ºC. Guzman *et al.* (2008) showed 30°C as ideal for lipase activity in a *B. pumilus* strain isolated from Laguna Verde, a saline environment.

In the present study for the *Pseudomonas putida* strain used carbon source was glucose. Banerjee *et al.,* (1985) also reported that some microorganisms showed higher activities when grown in medium containing glucose. Novotny *et al.,* (1988) reported that olive oil in combination with glucose increased the lipase activity. However in the few other cases the presence of olive oil,

together with glucose or glycerol in the medium significantly decreased both lipase and esterase levels. They also further inferred that, if olive oil was used as the only carbon source for growth, the enzyme activities of *Candida guilliermondii* and yeast showed a four to five fold increase. As reported by Nakashima *et al.,* (1988), the presence of olive oil as growth medium greatly enhanced the lipase activity of *Bacillus* strain. Rohit *et al. ,* (2001) reported that the lipase production was more when vegetable oil, olive oil, soya bean oil, sunflower oil and gingili oil were used as the carbon source.

In the present study, 0.2% of yeast extract was identified as the best nitrogen source was optimized. Fadiloglu band Erkmen, (2002) also reported that olive oil in combination with other nitrogen sources enhanced the lipase production, but in the presence of carbon source olive oil significantly ($P < 0.01$) decreased the lipase activity and biomass content. They also reported that organic nitrogen sources were found to increase lipase synthesis by *Candida rugosa* grown in the presence of olive oil.

In the present study the culture filtrate after membrane dialysis (i.e.) partially purified enzyme 85.0 U/mL/minute of activity showed. When cheaper source (i.e.) coconut oil cake at the rate of 10% was used instead of carbon and nitrogen source, surprisingly 147.0 U/mL/minute of enzyme was produced. Rashidah *et al.,* (2006) observed in PI12 lipase activities in both tributyrin and olive oil broth media at 5ºC were 0.75 U/mL and 0.017 U/mL. and the specific activities were 7.894 U/mg and 0.163 U/mg respectively. For the reaction at room temperature, the lipase activities in both tributyrin and olive oil broth media were 0.9 U/mL and 0.183 U/mL whereas the specific activities were 9.474 U/mg and 1.763 U/mg respectively.

Mohan *et al.* (2010) observed the lipase activity of *Bacillus* strains was high (0.0029 µg/mL/minute) when grown at the medium temperature of 37°C at the optimum pH of 7.0. Kanimozhi *et al.,* (2011) reported the maximum lipase activity by the *Bacillus* at 37°C and pH 7.0 where its production reached upto 0.01033 and 0.01066 µg/mL/minute. Among the different substrate the maximum activity was observed in gingili oil (0.01066 µg/mL/minute) at pH 7.0 and temperature 37°C.

In the present study SDS-PAGE analysis revealed that the protein profile of partially purified enzyme showed 34, 45 and 52 kDa proteins was obtained. From the literature, molecular weight of lipase produced by most bacteria such as *Pseudomonas aeruginosa* (Chihara-Siomi *et al.,* 1992; Chartrain *et al.,* 1993), *Pseudomonas putida* (Lee and Rhee, 1993), *Pseudomouas* sp. (Choo *et al.,* 1998; Yang *et al.,* 2000; Rashid *et al.,* 2001) was between 29 KDa – 45 kDa. However, there was also larger molecular weight of lipase isolated from different microbes. Thus the present study revealed many details on lipase enzyme produced by *Ps.putida* SBS037 strain.

The study proved that lipase producers are abundant in the sample collected from Vellar estuary. The potential Strain *Ps. Putida* SBS037 was selected as the most potential for lipase production.

Figure.1 Effect of pH on growth of *Pseudomonas putida* SBS037

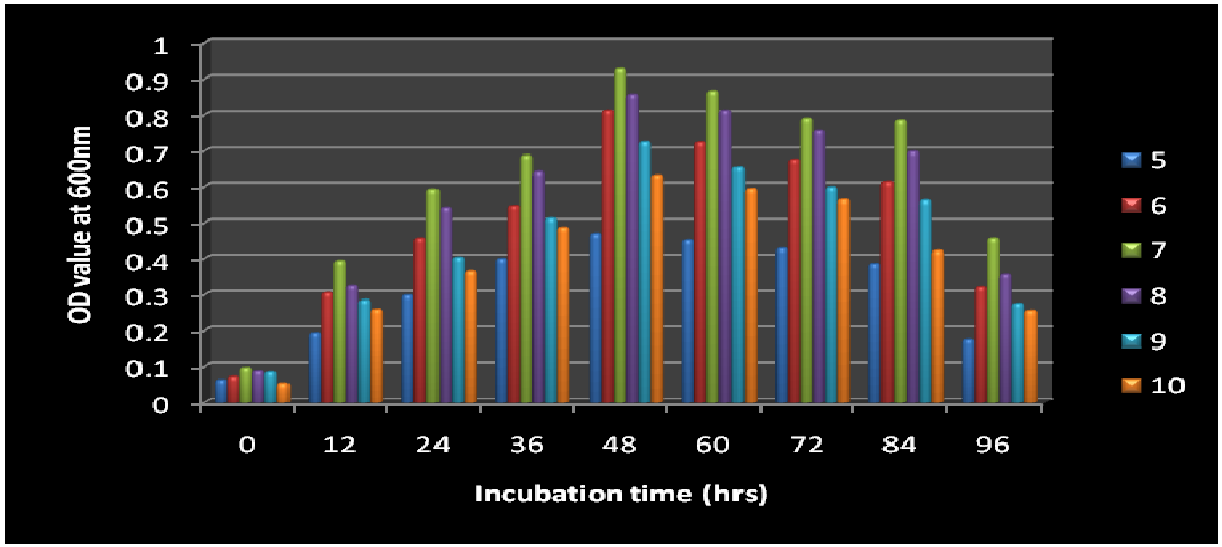


Figure. 2 Effect of Nacl concentration on growth *Pseudomonas putida* SBS037

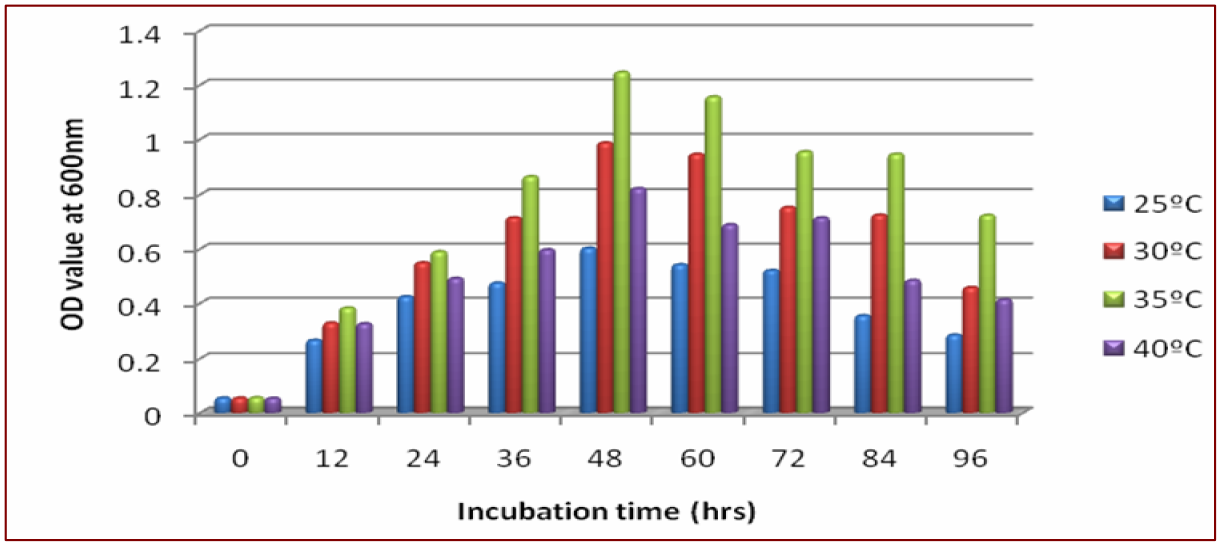


Figure. 4 Effect of temperature on growth *Pseudomonas putida* SBS037

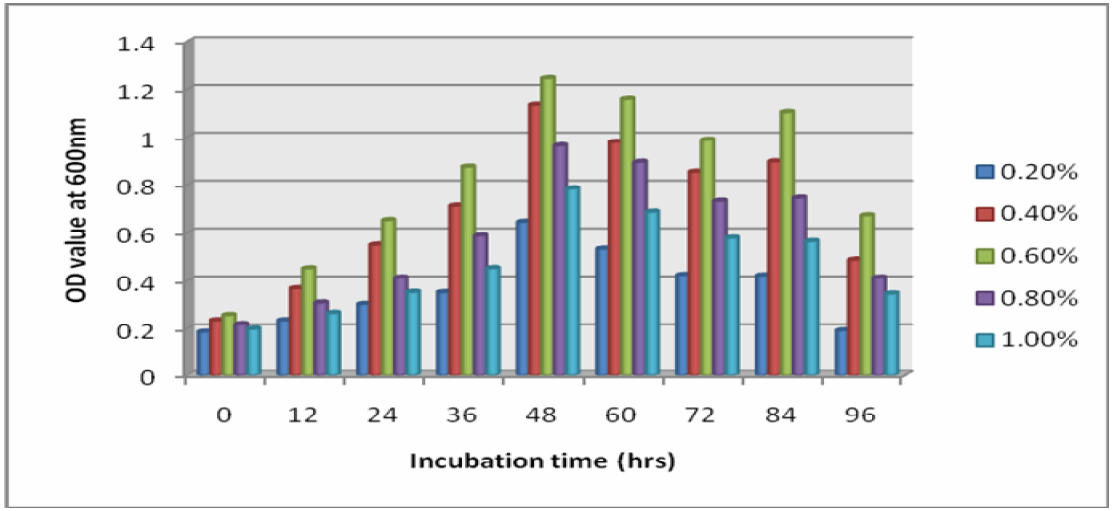


Figure.5 Effect of carbon sources on growth *Pseudomonas putida* SBS037

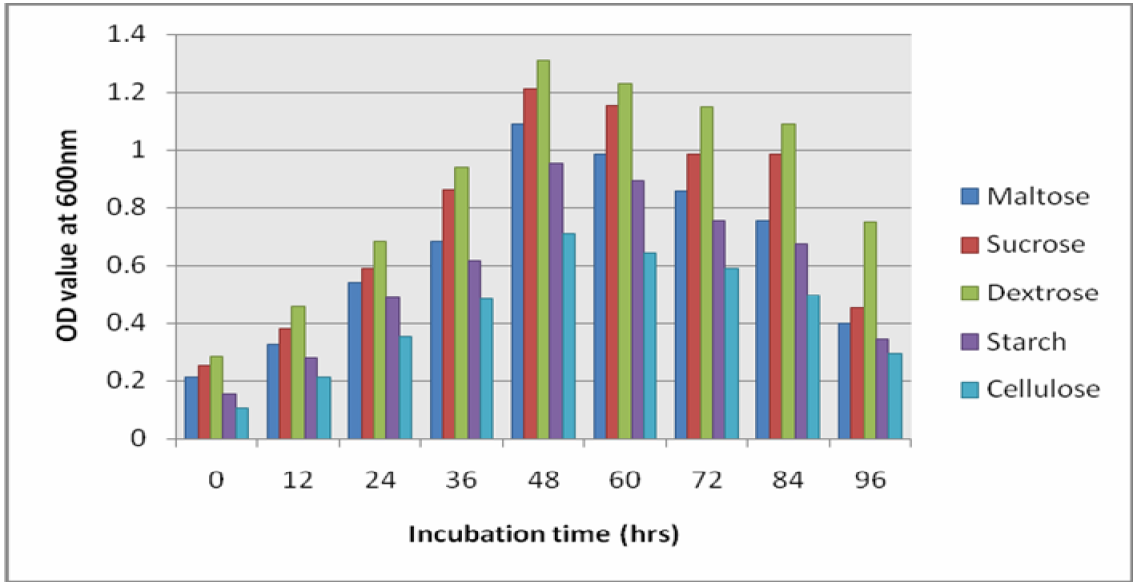


Figure.6 Effect of nitrogen sources on growth *Pseudomonas putida* SBS037

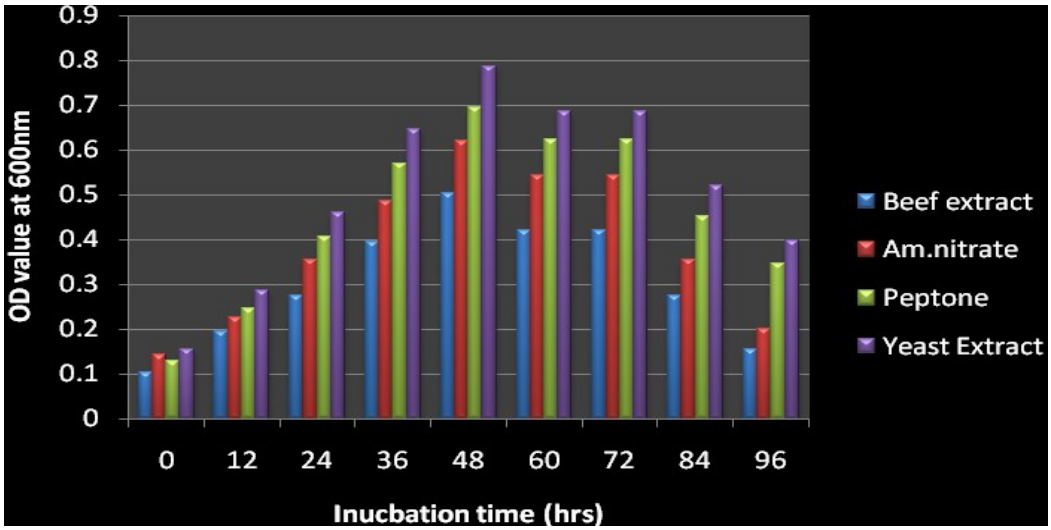
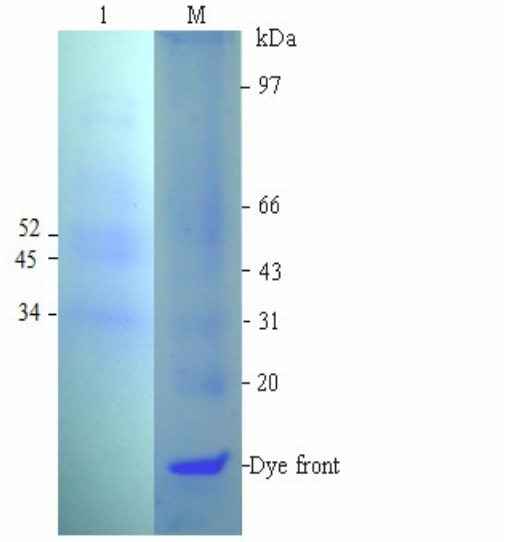


Figure.7 SDS-PAGE analysis of lipase enzyme



Lane 1: Sample Protein
Lane M: Standard Protein Molecular Weight Marker

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